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Departamento de Química

BEATRIZ BAPTISTA RIBAU

A EPIGENETICA NA LEUCEMIA LINFOCÍTICA AGUDA E CRÓNICA – POTENCIAL TERAPÊUTICO E AVALIAÇÃO DO PERFIL DE METILAÇÃO

EPIGENETIC IN ACUTE AND CHRONIC LYMPHOCYTIC LEUKAEMIA – THERAPEUTIC POTENTIAL AND METHYLATION PROFILE EVALUATION

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, com especialidade em Métodos Biomoleculares, realizada sob a orientação científica da Professora Doutora Ana Bela Sarmento Ribeiro, Professora associada com agregação da Faculdade de Medicina da Universidade de Coimbra, e do Professor Doutor Francisco Manuel Lemos Amado, Professor associado do Departamento de Química da Universidade de Aveiro.

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o júri

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palavras-chave

Leucemia linfocítica aguda (LLA); Leucemia linfocítica crónica (LLC); metilação do DNA; genes supressores tumorais; agentes hipometilantes; Azacitidina; Decitabina; inibidores das desacetilases das histonas; Panobinostat; Vorinostat

resumo

O cancro é uma doença multifatorial, provocada por múltiplas mutações do DNA e/ou alterações nos processos epigenéticos, que ocorrem durante o ciclo celular e que o organismo não consegue reverter.

A leucemia linfocítica aguda (LLA) é uma doença caracterizada por uma rápida proliferação de células percursoras da linhagem linfoide, imaturas, designadas de linfoblastos, que se acumulam na medula óssea e provocam declínio na produção de células normais.

A leucemia linfocítica crónica (LLC) é uma desordem caracterizada pela acumulação progressiva de linfócitos maduros resistentes à apoptose. É o tipo de leucemia crónica mais comum em adultos do sexo masculino com idade superior a 55 anos, sendo um evento raro em crianças.

Cada tipo de cancro é caracterizado não só por alterações genómicas recorrentes, mas também aberrações epigenéticas, demonstrando a sua importância no desenvolvimento destas patologias. As modificações epigenéticas são importantes para o silenciamento e/ou ativação de genes, fundamental para a diferenciação celular e de tecidos. As modificações epigenéticas mais estudadas no cancro são a metilação das ilhas CpG dos promotores de genes e as modificações pós-traducionais das histonas. Na LLA e na LLC, as duas modificações epigenéticas mais estudadas e associadas à sua patogénese são as alterações na metilação de DNA, como o caso da hipermetilação, e a desacetilação de histonas. Desta forma, os agentes hipometilantes e os inibidores das desacetilases das histonas são duas categorias de modeladores epigenéticos que estão em fase de estudo para possível utilização na terapêutica da LLA e LLC.

O presente estudo apresenta dois objetivos: avaliar o potencial terapêutico de dois agentes hipometilantes (Azacitidina e Decitabina) e de dois inibidores das desacetilases de histonas (Panobinostat e Vorinostat), em monoterapia e combinação, em duas linhas celulares de LLA-B (células 697 e KOPN8) e em células mononucleares de sangue periférico de doentes com LLC; avaliar o perfil de metilação das linhas celulares de LLA-B e das células mononucleares de LLC.

Desta forma, este estudo envolveu duas linhas celulares de LLA da linhagem B (células 697 e KOPN8) e 31 indivíduos (21 diagnosticados com LLC e 10 controlos não-neoplásicos).

As duas linhas celulares de LLA-B (células 697 e KOPN8) e as células mononucleares de sangue periférico de doentes com LLC foram incubadas com Azacitidina, Decitabina, Panobinostat e Vorinostat, em monoterapia (doses únicas e administração fracionada) e em combinação, durante 72h e 48h, respetivamente. O efeito citotóxico dos fármacos em estudo foi avaliado pelo ensaio de citotoxicidade de microcultura fluorométrica (FMCA). O estudo da morte e ciclo celular foram obtidos por Citometria de fluxo, com recorrência a ensaios de Anexina V/Iodeto de Propídio e Iodeto de Propídio/RNAse, respetivamente. Os padrões de metilação foram obtidos pela técnica MS-MLPA e os níveis de 5-mC foram determinados por Citometria, com recurso a marcação intracelular com anticorpos anti-5mC. Os anticorpos para deteção das proteínas CD5 e CD19 permitiram a distinção de linfócitos B normais ($CD5^-/CD19^+$), linfócitos B neoplásicos ($CD5^+/CD19^+$), linfócitos T normais ($CD5^+/CD19^-$) e outras células mononucleares ($CD5^-/CD19^-$), nos estudos de morte celular em amostras de LLC. O DNA utilizado nos estudos de metilação foi extraído das amostras de LLC e dos controlos pelo protocolo de extração por *salting out*.

A avaliação das diferenças entre as doses de monoterapia, os esquemas de combinação, o ciclo celular e os dados de morte celular foram determinados pela aplicação do teste estatístico não paramétrico Kruskal-Wallis (teste de comparações múltiplas de Dunn). Os dados obtidos pela técnica MS-MLPA foram analisados utilizando também o teste não paramétrico Kruskal-Wallis (teste de comparações múltiplas de Dunn) apenas nas linhas celulares. Nos doentes foi aplicado o teste de qui-quadrado (X^2). Um valor de probabilidade de $p < 0,05$ foi considerado estatisticamente significativo, tanto para as linhas celulares de LLA quanto para os estudos com doentes de LLC.

Os estudos *in vitro* demonstraram que os moduladores epigenéticos apresentam efeito citotóxico, reduzindo a viabilidade celular nas linhas de LLA-B, células 697 e KOPN8, sendo esse efeito dependente da concentração, tempo de incubação e tipo celular. As células 697 demonstraram ser mais sensíveis a todos os fármacos, uma vez que o IC_{50} foi alcançado com doses inferiores comparando com as células KOPN8, nas quais o IC_{50} não foi atingido com as doses testadas. As mesmas células demonstraram sofrer mais efeito quando tratadas com 5-AC do que com DAC. Os resultados obtidos das células KOPN8 demonstraram que as combinações não apresentam beneficio comparativamente à monoterapia. Por outro lado, nas células 697, as combinações de Panobinostat e Vorinostat com a Decitabina provocaram uma maior redução na viabilidade celular, comparando com os resultados obtidos na monoterapia, sendo independente dos esquemas de administração. O melhor esquema de administração demonstrou ser a administração de LBH589 e SAHA, 3 horas após DAC. Os quatro fármacos em estudo induziram morte celular por apoptose, tendo sido confirmada por alterações no aspeto morfológico das células. Também se observou um efeito anti-proliferativo, induzindo a paragem do ciclo celular na fase G_0/G_1. Adicionalmente, os fármacos 5-AC e DAC, em monoterapia e em combinação, induziram uma diminuição nos níveis de 5-mC. Os estudos do perfil de metilação demonstraram que nenhum dos fármacos, testados em monoterapia e combinação, provocaram a alteração no perfil de metilação de nenhum gene em ambas as linhas celulares.

Os estudos em LLC também demonstraram que os agentes hipometilantes e os inibidores das desacetilases de histonas reduziram a viabilidade celular dependente da dose. As combinações celulares não demonstraram benefícios em comparação com a monoterapia. 5-AC em combinação com LHB589 demonstraram melhores resultados que a monoterapia, provocando maior redução na viabilidade celular, contudo não foi estatisticamente significativo. Os esquemas de administração fracionada também não demonstraram benefícios, comparativamente à monoterapia. Ambos os agentes hipometilantes e os inibidores das desacetilases de histonas provocaram uma paragem na fase S do ciclo celular. Os quatro fármacos também demonstraram ter influencia apenas nos linfócitos B neoplásicos. Apenas as concentrações de IC_{50} dos dois inibidores das desacetilases de histonas (LBH589 e SAHA) apresentaram diferenças significativas na morte celular ($p<0,01$), induzindo mais apoptose que o controlo. Contudo, os dois agentes hipometilantes também provocaram um aumento da morte celular por apoptose nos linfócitos B neoplásicos. Os estudos de metilação demonstraram que os doentes com LLC apresentam níveis de metilação elevados dos promotores dos genes MSH6 (86%, 18/21), KLLN (67%, 14/21), WT1 (86%, 18/21) e GATA5 (71%, 15/21), quando comparados com o controlo, sugerindo o envolvimento da metilação no desenvolvimento da LLC.

Os resultados obtidos neste estudo sugerem que a metilação de genes supressores tumorais é um evento comum em doentes com LLC e que os modeladores epigenéticos induzem um efeito citotóxico dependente do tempo e da dose, reduzindo a viabilidade celular de células de LLA e LLC. Desta forma, os resultados são bastante promissores, encorajando estudos futuros.

keywords

Acute Lymphocytic Leukaemia (ALL); Chronic Lymphocytic Leukaemia (CLL); DNA methylation; Tumour supressor genes; DNA hypomethylating agents; Azacytidine; Decitabine; Histone deacetylase inhibitors; Panobinostat; Vorinostat

abstract

Cancer is a multifactorial disease caused by multiple unrepaired DNA mutations and/or epigenetic changes, occurred during cell life cycle, that the organism cannot detect and reverse.

Acute lymphocytic leukaemia (ALL) is a disease characterized by a rapidly proliferation of early lymphoid precursors (namely lymphoblast), arrested in an early stage of its development, that replace normal hematopoietic cells in the bone marrow and cause decline in the production of normal marrow cells. Chronic lymphocytic leukaemia (CLL) is a disorder characterized by a progressive accumulation of lymphocytes that are apoptosis resistant, being the most common type of chronic leukaemia found in adults and among men over 55 years, hardly affecting children.

Each type of cancer not only has its own recurrent genomic aberrations but also has its characteristic epigenetic changes which demonstrate its importance to cancer pathogenesis. Epigenetic modifications participate in the silencing and/or activation of genes that are the main key to cell and tissue differentiation. The most studied epigenetic changes in cancer are DNA methylation of CpG islands in regulatory regions of gene and post-translational modifications of histones. In ALL and CLL, aberrant DNA methylation, such as hypermethylation of CpG islands of gene promoters and abnormal histone deacetylation have been associated with cancer. Thus, DNA hypomethylating agents and histone deacetylase inhibitors are two categories of epigenetic therapies that are being studied as possible new therapies for ALL and CLL.

The present study has two main goals. First, evaluate the therapeutic potential of two DNA methyltransferase inhibitors (Azacytidine and Decitabine) and two histone deacetylase inhibitors (Panobinostat and Vorinostat), in monotherapy and in combination, in two B-ALL cell lines (697 and KOPN8 cells) and in peripheral blood mononuclear cells (PBMC) obtained from CLL patients. Second, evaluate the methylation profile of the B-ALL cell lines and CLL samples.

To this end, two B-ALL cell lines (697 and KOPN8 cells) and 31 individuals participated in this study, 21 patients diagnosed with CLL and 10 non-neoplastic controls.

ALL cell lines (KOPN8 and 697 cells) and PBMCs from CLL patients were incubated with 5-AC, DAC, LBH589, and SAHA, in monotherapy (single dose and daily administration) and in combination, during 72h and 48h, respectively. The cytotoxic effect of drugs was evaluated by fluorometric microculture cytotoxicity assay (FMCA). Cell death and cell cycle were analysed by flow cytometry through annexin V/Propidium iodide and PI/RNAse assays, respectively. Methylation patterns were determined by MS-MLPA and levels of 5-mC were determined by intracellular labelling with anti-5-mC antibodies by flow cytometry. CD5 and CD19 antibodies were used to identify normal B cells ($CD5^-/CD19^+$), neoplastic B cells ($CD5^+/CD19^+$), normal T cells ($CD5^+/CD19^-$) and other mononuclear cells ($CD5^-/CD19^-$). Methylation study was determined by MS-MLPA. For the methylation study, DNA was extracted from samples and controls by salting out protocol.

Abstract (cont.)

The evaluation of the differences between monotherapy doses, combination schemes, cell cycle and cell death data were determined by applying the nonparametric ANOVA Kruskal-Wallis test (Dunn's multiple comparisons test). MS-MLPA obtained data were analysed using nonparametric ANOVA Kruskal-Wallis test (Dunn's multiple comparisons test) for cell lines and chi-squared (X^2) test for patients. A probability value of $p<0.05$ was considered statistically significant for both cell lines and patient studies.

The *in vitro* studies showed that epigenetic modulators have a cytotoxic effect, being able to reduce cell viability in B-ALL cell lines, 697 and KOPN8. All four therapies demonstrated to have effect on cell viability that is concentration, incubation time and cell type dependent. 697 cells seem to be more sensitive to all four therapies, since the IC_{50} dose was reached with lower concentrations compared with KOPN8 cell line, where the IC_{50} was not reached with tested doses. In 697 cells, DAC demonstrated to have more effect than 5-AC, when comparing same doses. For KOPN8 cells, the combinations studied do not show beneficial results compared to those obtained in monotherapy. On the other hand, for 697 cells, the combination of LBH589 and SAHA with DAC leads to a higher reduction in cell viability compared to those observed in cells treated with drugs in monotherapy, independent of the administration scheme. The best administration scheme was the administration of LBH589 and SAHA, 3 hours after the administration of DAC. All four tested drugs induced cell death by apoptosis, confirmed by changes in morphologic aspects. It was also observed that those drugs, in a global way, they showed antiproliferative effect, inducing cell cycle arrest at G_0/G_1 phase. Moreover, 5-AC and DAC in monotherapy and in therapeutic combination, induced a decrease in 5-mC levels. Methylation data showed that none condition tested caused an alteration on gene promoter methylation levels, compared with control.

CLL studies also demonstrated that DNA hypomethylating agents and histone deacetylase inhibitors induced similar effects and all four therapies reduced cell viability in a dose-dependent manner. For the combination of therapies, none demonstrated to be statistically significant in comparison to monotherapy. 5-AC in combination with LBH589 obtained better results on cell viability than the ones obtained for monotherapy, causing an increased reduction on cell viability. However, this reduction was not significant. The results obtained for cells treated with the epidrugs, in a daily dose administration scheme, were very similar to the combination therapy, where none demonstrated to be statistically significant in comparison to monotherapy. DNA hypomethylating agent therapies and histone deacetylase inhibitors arrested cell cycle in S phase. Neoplastic B lymphocytes demonstrated to be more affected than normal B and T cells, which was expected. Only LBH589 and SAHA IC_{50} doses were considered statistically significant, compared with control, with $p<0.01$ for both conditions. However, all four therapies demonstrated to induce much more apoptosis on neoplastic B lymphocytes than on normal lymphocytes and other mononuclear cells. Methylation data showed that CLL patients had a significant higher methylation frequency of MSH6 (86%, 18/21), KLLN (67%, 14/21), WT1 (86%, 18/21) and GATA5 (71%, 15/21) gene promoters, when compared with controls, suggesting the involvement of DNA methylation on CLL development.

Results also suggest that the methylation of tumour suppressor genes is a common event in CLL patients and that epigenetic modulators induce a cytotoxic effect in cells, reducing cell viability in a time- and dose-dependent manner. Therefore, these results are promising and encourage further studies.

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List of abbreviations

5-hydroxymethylcytosine	5-hmC
5-methylcytosine	5-mC
Acute lymphocytic leukaemia	ALL
Acute lymphocytic leukaemia of the B-cell lineage	B-ALL
Acute myelogenous leukaemia	AML
Acute promyelocytic leukaemia	APL
Azacytidine	5-AC
Centro Hospitalar e Universitário de Coimbra	CHUC
Chronic lymphocytic leukaemia	CLL
Chronic lymphocytic leukaemia of the B-cell lineage	CLL
Chronic myelogenous leukaemia	CML
Chronic myelomonocytic leukaemia	CMML
Computerized tomography	CT
Decitabine	DAC
Dimethyl sulfoxide	DMSO
DNA methyltransferase enzyme	DNMT
European medicines agency	EMA
Fetal bovine serum	FBS
Fluorescein diacetate	FD
Fluorometric microculture cytotoxicity assay	FMCA
Food and Drug administration	FDA
Half maximal inhibitory concentration	IC_{50}
Histone acetyltransferase	HAT
Histone deacetylase inhibitors	HDACi
Histone deacetylase isoenzymes	HDAC
Hospital Distrital da Figueira da Foz	HDFF
Immunoglobulin heavy chain genes	IgHV
Isocitrate dehydrogenase	IDH
Methylation index	MI
Methylation-specific multiplex ligation-dependent probe amplification	MS-MLPA
Myelodysplastic syndrome	MDS
Multiple Myeloma	MM
Panobinostat	LBH589
Peripheral blood mononuclear cells	PBMCs

Phosphate buffered saline	PBS
Phosphatidylserine	PS
Polymerase chain reaction	PCR
Propidium Iodide	PI
Reduced representation bisulfite sequencing	RRBS
Roswell Park Memorial Institute 1640 medium	RPMI 1640
S-adenyl methionine	SAM
Sodium dodecyl sulphate	SDS
Suberoylanilide hydroxamic acid	SAHA
T-cell prolymphocytic leukaemia	T-PLL
Ten-eleven translocation 2	TET2
Ten-eleven translocation enzymes	TET
Vorinostat	SAHA
White blood cells	WBC
Whole-genome bisulfite sequencing	WGBS

1. Introduction

1.1.Cancer

Cancer is a disease caused by multiple unrepaired DNA mutations and/or epigenetic changes occurred during cell life cycle (1). Mutations take place in the organism but sometimes they can not be detected and be reversed during cell cycle checkpoints, tending to accumulate over the years. Those DNA alterations affect normal cell growth by altering the expression and/or function of, for example, proteins involved in cell cycle, cell division, DNA repair and apoptosis, being responsible for abnormal and uncontrolled proliferation of cells (2).

Despite cancer being designated a gene disease, carcinogenesis result from a combination of chemical, physical, biological and genetic factors (3). The process of cancer development has three stages: initiation, promotion and progression (Figure 1). The initiation results from an irreversible genetic alteration (one or more mutations, transversions, transitions and/or small deletions). The second stage of carcinogenesis is reversible and does not involve changes in the structure of DNA, only in promoter-receptor interactions for the genome expression. Normally in this stage, the altered cells (pre-neoplastic cells) proliferate uncontrollably. This stage is responsible for the transformation of a premalignant lesion to an invasive cancer. On the contrary, the last stage is irreversible and involves the growth of those clones selected previously, causing the fast increase in tumour size, and cells may undergo further mutations (3).

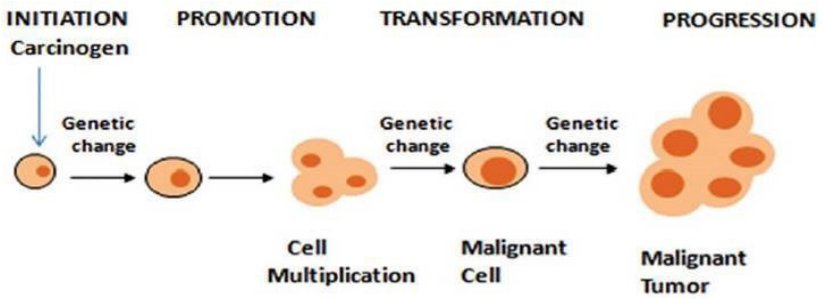

Figure 1 - Stages of cancer development: initiation, promotion and progression. Adapted from: (4)

In the next twenty years, it is expected that the incidence of cancer in Portugal increase in approximately 15% and, despite new treatments and new ways of diagnosis, it remains a major problem of public health (5). There are more than 100 types of cancer, being breast, prostate, lung, stomach and colorectal the most common in Portugal, according to "PORTUGAL Doenças Oncológicas em Números – 2015" from Direção Geral de Saúde (5). However, there are some less incident types of cancers such as haematological malignancies that, because of its aggressiveness and lack of effective treatments, require scientific attention.

1.2.Haematological Malignancies

Haematopoietic stem and progenitor cells in the bone marrow differentiate in many distinct types of mature blood cells, such as red blood cells, white blood cells (WBC) and platelets (6). Cancers that affect lymphoid lineage include acute lymphocytic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), lymphomas and multiple myeloma and the ones that affect myeloid lineage include acute myelogenous leukaemia (AML) and chronic myelogenous leukaemia (CML), myelodysplastic syndromes and myeloproliferative neoplasms (thrombocythemia, polycythemia vera and myelofibrosis) (Figure 2) (7).

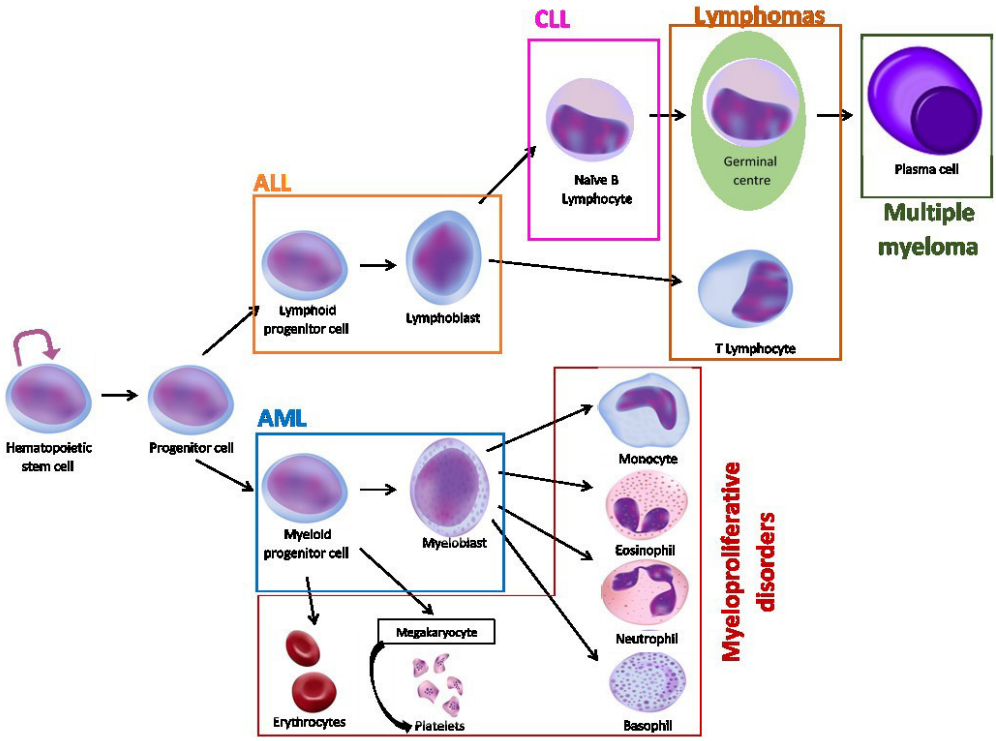

Figure 2 - Haematopoiesis and haematological malignancies. Stem cells have the capacity to renovate and originate progenitor cells that differentiate in lymphoid progenitor and myeloid progenitor cells. The last ones are responsible to differentiate and create the different types of leucocytes, red blood cells and platelets. Acute lymphocytic leukemia, ALL; Chronic lymphocytic leukemia, CLL; Acute myelogenous leukemia, AML; Chronic myelogenous leukemia, CML.

Leukaemia results from many aberrant alterations of blood-forming cells from the bone marrow, causing them to lose the ability to properly mature, thus affecting its function and in some cases cause an increase of proliferation rate and/or resistance to cell death (8). This type of cancer is sub-classified by the type of blood cell that is affected, as mentioned before, and by the stage of the development of cells, acute if it affects immature cells or chronic if it affects mature cells (9). The present study focused on two types of leukaemia of the lymphoid lineage, ALL and CLL.

1.2.1. Acute lymphocytic leukaemia (ALL)

ALL is a disease characterized by a rapidly proliferation of early lymphoid precursors (namely lymphoblast cells), arrested in an early stage of its development, that replace normal hematopoietic cells in the bone marrow and cause decline in the production of normal marrow cells (8). Leukaemia lymphoblast cells also proliferate in other

organs, such as liver, spleen and lymph nodes. ALL has its origin from genetic abnormalities that can occur *in utero* and are responsible for the transformation of lymphoblast cells into leukemic B and T cells (9). ALL is an heterogenous disease and include T-cell ALL and B-cell ALL.

ALL is the most common type of cancer and particularly in children (80-85% of cases worldwide of ALL), with and increased incidence in ages between 2 and 5, and is somewhat more common in males than females (10). In children, ALL is the most common leading cause of cancer-related death. Adulthood ALL is a rare disease with high mortality. ALL has an annual incidence of 2/100000 in children and 0.7/100000 in adults (8). Patients with ALL are categorized in three prognostic groups; good risk, intermediate risk and poor risk, according to characteristics such as cytogenetics, age and WBC count and patients with precursor B-cell ALL have extremely poor prognosis (11).

There are some genetic syndromes that predispose to ALL such as Down syndrome, Fanconi anaemia, Bloom syndrome, ataxia telangiectasia and Nijmegen breakdown syndrome, but represent a minority of cases. The exposure to certain viruses like Epstein-Barr and Human Immunodeficiency virus may also promote ALL development (8). However, most cases are a *de novo* malignancy with no associated cause (12).

ALL diagnosis include blood and bone marrow tests (morphology, molecular biology and flow cytometry tests), imaging tests such as X-ray, computerized tomography (CT) or ultrasound scan, and spinal fluid test, giving information about cancer localization and if it has spread to other organs (13). Treatment passes through chemotherapy, targeted drug therapy, radiation and stem cell transplant. Normally, older adults have worse prognosis than children with ALL. Failure to respond to chemotherapy is very frequent and normally those patients do not respond to other chemotherapy associated regiments, demonstrating how important it is to develop new therapeutic strategies for those patients (14).

ALL is a genetic disease, since it results from acquired genetic mutations (somatic) that promote proliferation, survival and/or impaired differentiation of the lymphoblast cells (8). ALL patients may present three types of recurrent genetic aberrations: numerical abnormalities (gain or loss of whole or segments of chromosomes), chromosomal translocations and other molecular genetic abnormalities. Most cases present chromosomal aberrations that result in distinct ALL subtypes, each with individual characteristics (9). Some of these abnormalities, particularly chromosomal translocations, have diagnostic value which may impact prognosis or treatment. Hyperdiploidy and hypodiploidy can also be detected in ALL patients.

There are some translocations frequently identified in patients with ALL, such as t(12;21) (p12;q22) TEL AML 1 [ETV6-RUNX1], t(5;14)(q31;q32) IL3-IGH, t(1;19) (q23;p13.3); E2A-PBX1 [TCF3-PBX1], t(9;22)(q34;q11.2) [BCR-ABL1] and t(v;11q23) with rearrangement of MLL, that may be used for the diagnose, however, individually, they can not be used as the cause of leukaemia (15). Those chromosomal translocations cause abnormal expression of genes that may be responsible of the immature cells arrest, characteristic of ALL. Hyperdiploidy with 51-65 chromosomes and t(12;21)(p13;q22) are associated with good risk and is predominantly

observed in children. On the other hand, translocation t(9;22)(q34;q11) is characteristic of poor-risk and is predominantly observed in adults. The frequency of those abnormalities varies among populations due to ethnicity and geographic factors (15).

Despite all the information and therapies, ALL is still a cause of death in many children and because of its aggressiveness it is important to continue researching, develop new cancer treatments and new ways of using existing treatments to promote a better life for patients.

1.2.2. Chronic lymphocytic leukaemia (CLL)

CLL is a neoplastic disorder characterized by a progressive accumulation of lymphocytes that are apoptosis resistant (16). There are two subtypes of CLL based on the subtype of lymphocyte that is affected: B-cell CLL, the most common, affecting more than 95% of people with CLL; and T-cell CLL, now called T-cell prolymphocytic leukaemia (T-PLL), that is extremely rare and aggressive (17). B-cell CLL is a slow growing leukaemia that affects B-cells in their development pathway (18). Normal B-cells produce immunoglobulins that protect the body against infections and other diseases but in CLL patients B-cells do not mature properly and lost their immune function. CLL cells gain the ability to live longer and start to accumulate mostly in the bone marrow, passing then to blood stream, lymph nodes, spleen and liver. The accumulation of abnormal cells in the bone marrow interfere with normal blood cell production reducing the number of red blood cells produced, which increase anaemia, infections, bruises and bleeding cases in CLL patients (19).

CLL is the most common type of chronic leukaemia found in adults and among men over 55 years, hardly affecting children (20). Clinical evolution of CLL is extremely variable and heterogeneous, and normally remains undetectable, without associated symptoms, for many months or even years. Patients may live with the disease without having any impact in their health and lifestyle. However, sometimes patients present some symptoms such as asthenia, anorexia, loss of weight, fever (>38˚C) nocturnal sweats, enlarged lymph nodes, abdominal pain, hepatomegaly and splenomegaly (21). In some diagnosed cases, CLL patients does not require treatment and can survive for many years, normally dyeing from other causes (22). In other cases, the disease is more aggressive and treatment is required (23).

The causes of CLL are unknown, making it a difficult disease to prevent. However, the genes that control blood cell development seems to play an important role in its development and progression (18). There are some risk factors associated with this disease, such as age, race, gender, and exposure to certain chemicals (24). Besides, the development of new targeted drugs for CLL, all treatments have side effects and some patients are resistant to conventional and new therapies, so its choice need to be based on the stage and type of cancer (25). Stem cell transplant can also be done in younger patients (26). Typically, CLL patients also take supportive therapy to reduce the pain and treat the symptoms and receive blood and platelets transfusions to restore their levels (20). Despite all current treatments, CLL remains an incurable disease.

Approximately 80% of CLL patients have some cytogenetic abnormality that can be used in prognosis like deletions in chromosomes 13 (del 13q14 – 50%), 17 (del 17p13 – 10%) and 11 (del 11q22 – 20%) and trisomy of chromosome 12 (20%) (27,28). Patients with del(13q14) have good prognosis whereas those with del(17p13), involving P53 pro-apoptotic gene, have bad prognosis because they do not respond well to conventional therapy and tend to have a rapidly evolving disease (29,30).

Somatic mutations of variable segments of immunoglobulin heavy chain genes (IgHV) that occur during B-cell development are also important to distinguish two patient groups, mutated IgHV patients (60%) and unmutated IgHV patients (40%) (31). Normally, patients with mutated IgHV also have del(13q14) and consequently develop a less aggressive disease having good prognosis. On the contrary, unmutated IgHV patients have frequently trisomy 12 which is associated with a worse prognosis (32). In those cases, CLL cells are more responsive to BCR signalling and other microenvironmental survival signals turning them resistant to external apoptotic signals (33).

CD38 and protein ZAP-70 are some good prognostic biomarkers (34). They are associated with poor prognosis and correlates with mutations of IgHV, being highly expressed in unmutated IgHV cells (35). CD38 was the first marker to be associated with IgHV mutations, being nowadays considered an independent prognostic biomarker. Presently, ZAP-70 biomarker is extremely useful as prognostic factor because its expression remains stable and is indicative of disease progression and survival (36). Patients with CLL also display elevated levels of some cytokines such as CCL3 and CCL4 in the plasma, making them also good prognostic biomarkers (37). Despite all the characterization of CLL, its pathogenesis is still under investigation because of its complexity and heterogeneity.

1.3.Epigenetic modifications

Comprehensive analyses of various cancers have showed that each type of cancer, not only has its own recurrent genomic aberrations, that consequently cause alteration of some gene expression products and alteration of the apoptotic signalling pathway, but also has characteristic epigenetic changes, demonstrating its importance to cancer pathogenesis (38).

Pioneering work on epigenetics field was carried out by Miescher, Fleming, Kossel and Heitz, between 1869 and 1928, where they defined nucleic acids, chromatin and histone proteins, leading to the cytologic distinction of euchromatin and heterochromatin (39). The term “epigenetics” was first used in 1942 by Conrad Waddington to refer to the genome-environment interactions responsible for the development and differentiation of more complex organisms, being responsible for the observable variety in tissues such as skin, liver and brain (40). Waddington defined epigenetics as changes in phenotype without changes in genotype. Nowadays, the term is used to refer to heritable and reversible alterations that, unlike genomic abnormalities, do not cause alterations of the genomic sequence, it only affects gene expression patterns by adapting chromatin (41). Early in 1948, it was first detected chemical modifications of DNA bases and its role for DNA methylation. In the three-quarters of a century later, technological advances, such as chromatin immunoprecipitation, next generation sequencing and DNA sequence

analyses, have enabled the analysis of the epigenome at base-pair resolution and have allowed epigenomic profiling of normal and altered cells giving new insights into disease processes (39). As referred before, epigenetic modifications are reversible which offers a considerable promise for therapies based on the adaptive nature of epigenetic control.

Epigenetic changes are a regular and natural process but can also be influenced by many factors including age, lifestyle and environment (38). Majorly, those modifications are very important for the organism but sometimes they can be involved in the development of some diseases such as cancer, since the information carried by epigenetic modifications plays a very important role in many processes involving DNA such as transcription, DNA repair and replication. Consequently, abnormal pattern expression of DNA or alterations in chromatin regulators may lead to cell abnormalities and development of tumours.

The most studied epigenetic changes in cancer are DNA methylation of CpG islands in regulatory regions of gene, dating back to studies from Griffith and Mahler in 1969, post-translational modifications of histones and microRNAs (42). Epigenetic modifications participate in the silencing of genes that are the main key to cell and tissue differentiation. Only half of the approximately 25000 protein-coding genes that constitute the mammalian genome are expressed in any individual cell, some are ubiquitously expressed, but others are active in some types of cells or exhibit different expression patters from cell to cell (43). Normally, gene inactivation is preceded by DNA methylation and repressive histone alterations which cause condensation of DNA and consequent inability of the transcriptional machinery to bind to DNA and express specific genes (Figure 3) (42). Inversely, gene activation results from activating histone alterations and lack of DNA methylation. DNA and chromatin manipulation by epigenetics relies on the regulation of the epigenetic machinery like DNA methyltransferase enzymes (DNMTs), histone modification enzymes, methyl-binding proteins, miRNAs, among others.

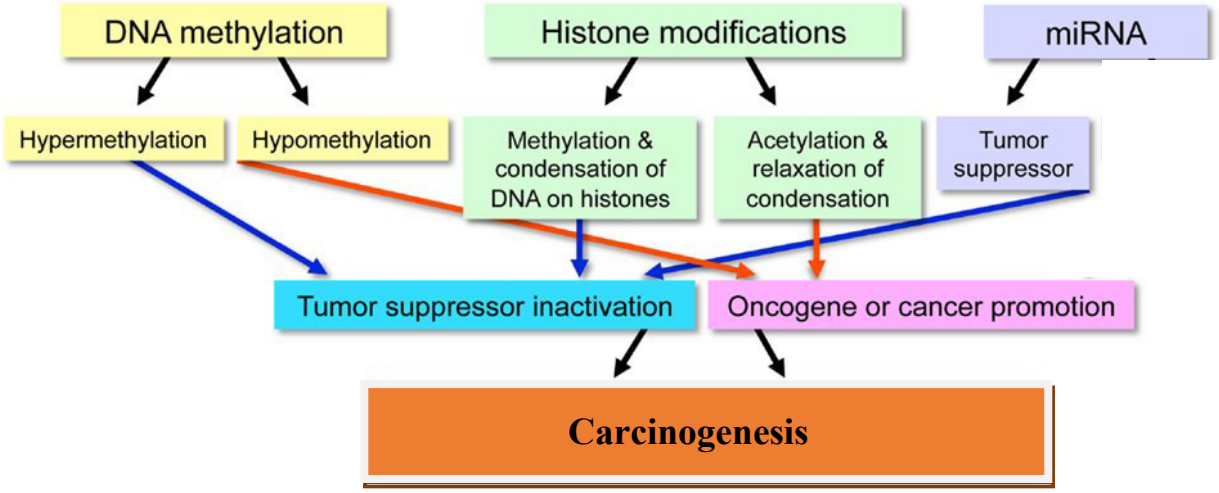

Figure 3 - Epigenetic modifications and the effect on tumour suppressor genes and oncogenes. The activation of oncogenes and the inhibition of tumour suppressor genes are responsible for the development of cancer. Adapted from: (44)

1.3.1. DNA methylation

DNA methylation is an epigenetic mark that regulates gene expression, being the first epigenetic modification discovered and studied (45). DNA cytosine methylation is a normal process that occurs and regulates nuclear structure and gene activation in processes such as cell differentiation, X chromosome inactivation, tissue specific expression, genomic imprinting, transposon silencing, gene regulation and tissue aging (46). This process of transferring a methyl group from S-adenyl methionine (SAM), enzymatic cofactor, to the 5-carbon of a cytosine near to a guanine, to form 5-methylcytosine (5-mC), is catalysed by DNMTs, with DNMT1, DNMT3A, and DNMT3B being the fundamental DNMTs in mammalian cells (Figure 4). The role of 5-mC on gene regulation was proposed in the mid-1970s by Holliday and Pugh but the association of DNA methylation with gene repression was established only in 1980, as well as the existence of CpG islands (39).

The current human genome assembly contains approximately 3 x 10^7 CpG dinucleotides that can exist in the methylated or unmethylated state making it difficult to analyse statistically. Edwards *et al*. (2016) analysed the genome by whole-genome methylation profiling and concluded that 75% of all promoters are within CpG islands and are unmethylated and the remaining 25% have very low CpG densities and the methylation process does not seem to regulate their expression (47). Researchers also observed that many CpG islands are not associated with promoters or other regulatory sequences and the consequences of their methylation are unknown and probably with inconsequential biological significance.

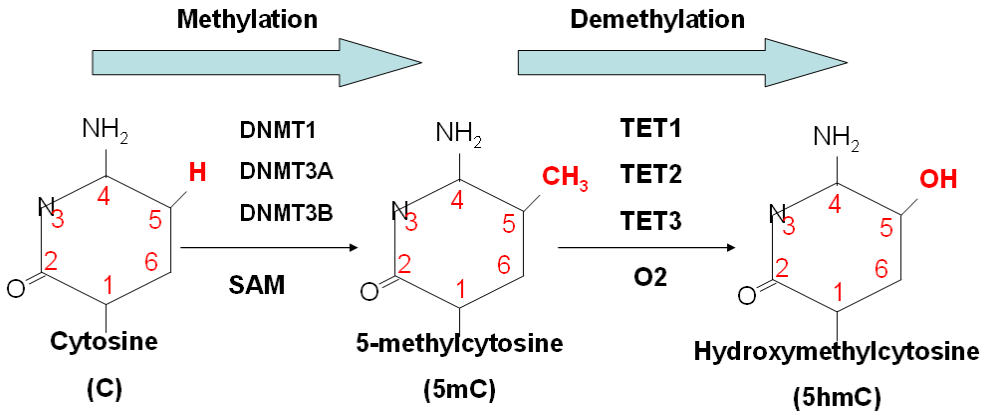

Figure 4 - Schematic diagram representing genomic DNA methylation and demethylation in cytosine. DNMT1 3A and 3B enzymes are involved in the process of methylation and TET1, 2 and 3 enzymes are responsible for the reverse process. DNA methyltransferase, DNMT; S-adenyl methionine, SAM; ten-eleven translocation, TET; cytosine, C; 5- methylcytosine, 5-mC; 5-hydroxymethylcytosine, 5hmC; Oxygen, O_2. Adapted from: (48)

The enzymes that participate in the methylation process are classified as writers that catalyse the addition of methyl groups onto cytosine residues (DNMTs), erasers that modify and remove the methyl group (TET1, 2 and 3 as other enzymes), and readers that recognize and bind to methyl groups, influencing gene expression (MBD proteins, UHRF proteins and the zinc-finger proteins) (49). Methyltransferases DNMT3A and DNMT3B are designated *de novo* because they can methylate hemi-methylated and unmethylated CpG at the same rate (Figure 5a) (50). DNMT1 is responsible to maintain the methylation patters of DNA through cell cycle (during DNA

replication), predominantly methylation of the hemi-methylated CpG di-nucleotides in the mammalian genome (Figure 5b). The ten-eleven translocation enzymes (TET) add a hydroxyl group onto the methyl group of 5-mC to form 5-hydroxymethylcytosine (5-hmC) that can then be converted back into cytosine in mammals (Figure 4). Therefore, these enzymes play an important role in transcriptional repression of genes for cell differentiation and haematopoiesis (51).

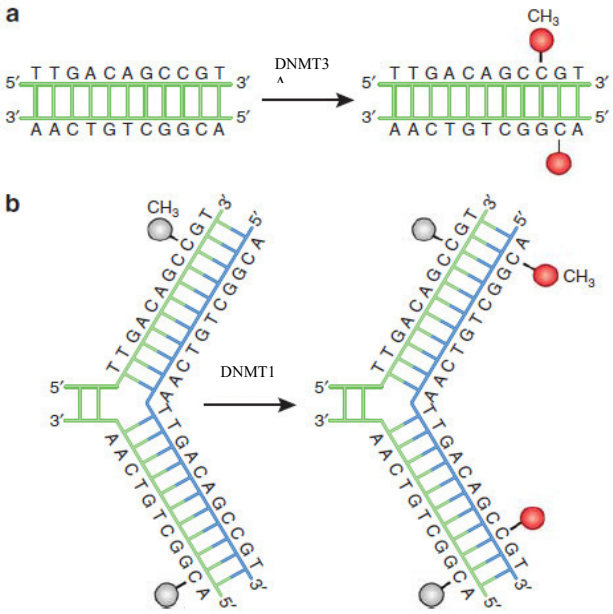

Figure 5 - DNA methylation pathways. DNA methyltransferase enzymes (DNMTs) catalyze the process of transferring a methyl group from S-adenyl methionine (SAM) to the 5-carbon of a cytosine near to a guanine), with DNMT1, DNMT3A, and DNMT3B being the fundamental DNMTs in mammalian cells. (a) DNMT3A and DNMT3B are de novo DNMTs and transfer the methyl groups (red) onto naked DNA. (b) DNMT1 is the maintenance DNMT and maintains DNA methylation patterns during replication (grey) and adds new methyl groups (red) to the new replicated strand (blue). Adapted from: (49)

Over the last three decades, there has been an increase of aberrant DNA methylation studies in haematological malignancies, not only to understand how the methylation of promoter genes alter the expression of genes, but also to develop targeted therapies (52). Recent studies show that aberrant DNA methylation, including hypomethylation and hypermethylation of CpG islands of gene promoters, are important for the progression of some tumours and there are strong relations between DNA methylation and transcriptional inactivation, which result in the functional equivalent of genome deletions and inactivating mutations (38,53). Cancer is now recognized as an epigenetic disease, since it was demonstrated that cancer cells suffer alterations on their genomic methylation patters including hypomethylation and hypermethylation of gene promoters when compared with normal cells (Figure 6) (9).

Repetitive sequences are generally methylated at cytosine nucleotides in normal cells, however in cancer cells it is observed a global loss of methylation that leads to chromosomal instability and activation of endoparasitic sequences (54). CpGs located on gene bodies are usually methylated in normal cells, inactivating gene expression, but in cancer cells this pattern is reversed, causing initiation of the transcription process at several incorrect sites. On the other hand, CpG islands in promoter sequences are typically unmethylated in normal cells and in cancer cells they can be hypermethylated, leading to transcriptional repression. The same patterns are seen in CpG island shores, located upstream of promoters. To conclude, Figure 7 shows what happens when the promoter is not

methylated, as observed in the CpG islands of genes in normal cells, where it promotes gene transcription. However, in cancer cells the transcription of genes are blocked by methylation of the CpG islands, not being recognized by polymerase enzymes.

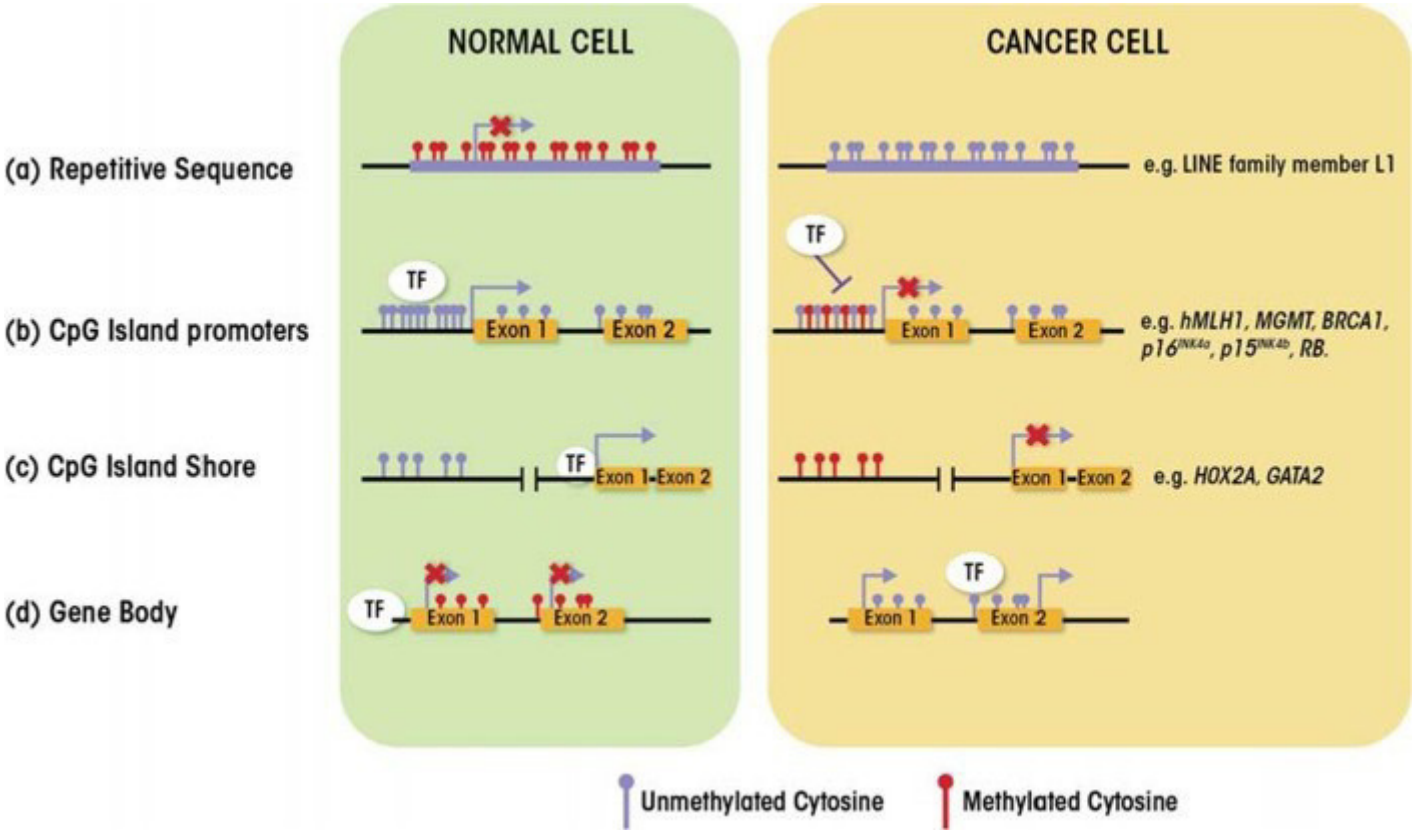

Figure 6 – DNA methylation patterns in normal and cancer cells. In normal cells, repetitive sequences and gene body are normally methylated, and CpG islands are unmethylated. However, in cancer cells this pattern is reversed and CpG are very affected by methylation. Transcription factor, TF. Adapted from (54)

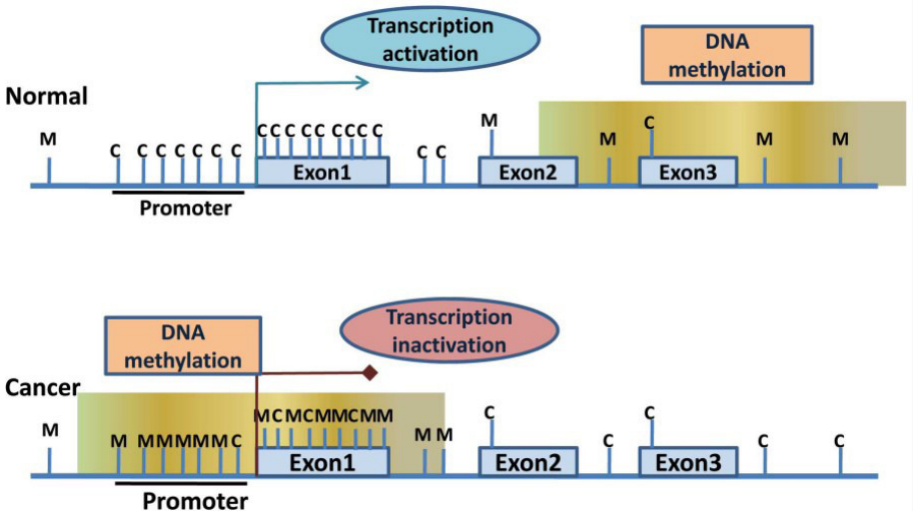

Figure 7 - Global changes in normal and cancer cells. In normal cells CpG islands are not methylated allowing transcriptional activation. In cancer cells are observed reverse patterns. Methylated, M; cytosine, C. Adapted from (55)

Remarkable progress has been made during the past few decades on DNA methylation, however the role of epigenetic events in cancer has not been fully explained. Nevertheless, it is already known that the methylation

patters in cancer cells are right the opposite of normal cells constituting a great starting point to the development of more efficient therapies to reverse this process that the organism tends to induce in neoplastic cells.

1.3.1.1. DNA methylation of CpG promoter islands of genes in ALL

The molecular mechanisms responsible for the transformation of lymphoid progenitor cells into ALL cells are currently unknown. However, epigenetic changes such as DNA methylation, in combination with somatic mutations and other genetic aberrations, may influence its transformation into malignant cells (56). Aberrant epigenetic lesions, particularly DNA methylation of CpG promoter islands of genes, are very common in ALL as established in recent data from multiple laboratories indicating that several hundred genes are supressed by methylation in ALL (57). In ALL patients, genes methylation has been associated with relapse, poor prognosis and lower survival (58).

The study of epigenetic alterations in ALL or in other types of cancer and disease may allow to identify patients with poor prognosis, when treated with conventional therapy, to develop new techniques to evaluate residual disease, to understand the differences between paediatric and adult ALL and to choose new therapeutics based on hypomethylating agents. Initial studies of DNA methylation in ALL involved the analysis of single genes in limited number of samples, focusing on genes such as *calcitonin*, *TP15, TP73, E-cadherin, ER, DKK-3, LATS2/KPM, HCK, DBC1, BNIP3*, among others (9,57). However, it is apparent that human cancer is characterized by the concomitant methylation of multiple genes. An initial study in adult ALL, analysed 10 genes (*MDR1, THSBS2, THSBS1, MYF3, ER, TP15, CD10, c-ABL, TP16, TP73*) in a cohort of 80 patients and demonstrated that 85% of these patients had methylation of at least 1 gene and 40% of patients had 3 or more genes methylated (59). CD10 expression demonstrated to be inversely associated with methylation of *CD10* gene and methylation of c-*ABL* gene was only observed in patients with Philadelphia chromosome alterations. Researchers also associated a bad prognosis with methylation of *TP73* and *TP15* genes. In another study, it was analysed 15 genes in more than 250 patients, both adult and paediatric ALL, and were obtained similar results, where 77% of patients showed to have at least 1 gene methylated, 35% of patients had methylation of 4 or more genes and increased number of methylated genes was associated with worse outcome (60). DNA methylation of 69 paediatric B-ALL and 42 non-leukemic control samples were analysed and revealed 325 genes hypermethylated and down regulated and 45 genes hypomethylated and upregulated, unrelatedly to ALL subtypes (61).The results obtained in this type of studies confirm the prevalence and clinical relevance of aberrant DNA methylation in ALL.

As mentioned before, methylation of multiple genes is very common in ALL and since methylation can supress tumour suppressor genes, it is very possible that those genes may confer distinct clinical pathological characteristics and worse prognosis. In a study, researchers analysed 3 genes (*TP15*, *TP73* and *TP57*) that showed prognostic value; *TP57* showed to be methylated in approximately 50% of adult ALL patients and displayed correlation with the other genes *TP73* (*TP53* homologue) and *TP15* (62). Patients with methylation of more than one of those genes demonstrated worse prognosis that patients with no methylation or methylation of 1 gene, because these three tumour suppressor genes have a role in cell cycle regulation and a slight alteration of their

expression may alter cell survival, as seen in ALL. The methylation of multiple genes is associated with worse prognosis and gets worse with the increase of methylated genes number (60).

Since epigenetic alterations are reversible, the genes silenced by hypermethylation can be reactivated by recurring to hypomethylating therapies. In a large-scale methylation study, Taylor et al identified 262 methylated genes in ALL, being 11 genes (*DCC, DLC-1, DDX51, KCNK2, LRP1B, NKX6-1, NOPE, PCDHGA12, RPIB9, ABCB1*, and *SLC2A14*) identified as differentially methylated between ALL patients (63). Researchers also found that the methylation status of *DDX51* differentiated B- and T-ALL subtype patients. The relationship between methylation and expression of genes was also studied in ALL cell lines treated with hypomethylating agents (5-aza-2-deoxycytidine and Trichostatin A). After treatment, ALL cell lines presented increased mRNA expression of some methylated genes, demonstrating that the administration of hypomethylating agents can represent a new therapy to treat ALL patients (64). In Table 1 are summarized a list of genes already studied and identified as being affected by methylation in ALL patients, demonstrated how ALL is very heterogenous and complex, since different signalling pathways seem to be affected by methylation.

Table 1 - Genes known to be methylated in ALL.

Gene	Chromosomal location	Function	Number methylated/Number analysed	% (Range)	References
RSPO1	1p34	WNT signalling	46/46	100	(66)
GIPC2	1p31	Prostanoid signalling	31/31	100	(66)
TP73	1p36	Transcription factor	11/35, 17/80, 45/251	18–31	(59,60,67,68)
KCNK2	1q41	K+ channel	14/16	87	(64)
LRP1B	2q21	LDL complex	15/16	93	(64)
CD302	2q24	GTPase negative regulator	11/16	68	(64)
ERC2	3p14	CNS, synapsis	49/57	86	(66)
MAGI1	3p14	Guanylate kinase	27/45	60	(66)
ADCY5	3q13		38/56	68	(66)
MME	3q25	Peptidase	8/80	10	(59)
HSPA4L	4q28	Heat shock protein	24/35	69	(66)
SFRP2	4q31	WNT signalling	42/261	16	(69)
OCLN	5q13	Tight junction	31/41	76	(66)
EFNA5	5q21	Ephrin signalling	44/58	76	(66)
SMSX2	5q34	Transcriptional repressor	54/55	98	(66)
GFPT2	5q34	Aminotransferase	8/35	23	(66)
LATS1	6q24	Kinase	100/251	40	(60)
ESR1	6q25	Estrogen receptor	17/18, 29/80	36–94	(59,70)

Table 1 - Genes known to be methylated in ALL. (continuation)

PRKN	6q26	Proteasome degradation	67/251	27	(60)
THBS2	6q27	Cell adhesion	42/80	52	(59)
SFRP4	7p14	WNT signalling	55/261	21	(69)
ABCB1	7q21	Transmembrane transporter	36/80	45	(59)
SFRP1	8p12	WNT signalling	99/261	38	(69)
CDKN2B	9p21	Cell cycle control	17/45, 20/46, 17/34, 19/80,73/251	23–50	(59,60,68,71,72)
GNA14	9q21	G protein	27/44	59	(66)
BRINP1	9q33	Inhibitor of SIRT1	29/170	17	(73)
ABL1	9q34	Kinase	6/80	8	(59)
DAPK1	9q34	Apoptosis	33	13	(60)
PTEN	10q23	Phosphatase	50	20	(60)
SFRP5	10q24	WNT signalling	73	28	(69)
BNIP3	10q26	Apoptosis	5/34	15	(74)
CALCA	11p15	Calcium metabolism	6/7, 13/14, 44/47, 45/105	42–93	(75–78)
DKK3	11p15	WNT signalling	60/183	33	(69)
CDKN1C	11p15	Cell cycle control	31/63, 45/251	18–50	(59,60)
SLC2A14	12p13	Glucose transport	12/16	75	(64)
WIF1	12q14	WNT signalling	78	30	(69)
APAF1	12q23	Apoptosis	85	34	(60)
DDX51	12q24		8/16	50	(64)
DACT1	14q23	WNT signalling	68/261	26	(69)
THBS1	15q15	Cell adhesion	16/80	20	(59)
IGDCC4	15q22		13/16	81	(64)
SALL1	16q12	Zn finger protein	41/41	100	(66)
CDH1	16q22	Cell adhesion	18/33, 92/251	37–54	(59,79)
CDH13	16q23	Cell adhesion	87/251	35	(60)
DCC	18q21	Putative tumour suppressor gene	14/16	87	(64)
ZNF382	19q13	Zn finger protein	24/46	52	(66)
KLK10	19q13	Serine protease	143/251	57	(60)
HCK	20q11	Kinase	9/44	20	(80)
SERINC3	20q13		22/251	9	(60)
MN1	22q12	Involved in meningioma	45/53	85	(66)

Researchers are investigating if the differences in prognosis between adult and paediatric ALL could be related, in part, to DNA methylation. The first study demonstrated that there were no obvious differences in the frequency of methylation in adult and paediatric ALL (65). However, the study is inconclusive because paediatric

ALL patient sample was small. In another study, researchers demonstrated not only that methylation is very frequent in adults and children, but also that the aberrant methylation of multiple genes is common in paediatric ALL, being correlated with prognosis, but not explaining why ALL is more aggressive in adults (60). One difference that may explain the aggressiveness of ALL in adults compared with children ALL may be the type of pathway affected by methylation. One example is *TP73/TP15/TP57* pathway, previously refereed, that can be inactivated by methylation and is observed in approximately 25% of adult patients and is extremely rare in younger patients (65).

1.3.1.2. DNA methylation of CpG promoter islands of genes in CLL

DNA methylation of CpG promoter islands of genes is also very frequent in CLL patients. Rush *et al* (2004) analysed 10 primary CLL samples and demonstrated that CLL is characterized by extensive CpG island methylation relative to normal B lymphocytes and the percentage of CpG islands to be aberrantly methylated relative to healthy donors were between 2.5–8.1% (81). In the same study, researchers identified 193 genes or sequences that are novel targets for methylation in CLL. Some of those genes were transcription factors (*DERMO1, FOXE1, TBX3* and *IPF1*), others were involved in the nervous system functions (*TBR1, GLRB* and *PAK5*) and it was also identified the *GRM7* gene that appears to inhibit cyclic AMP signalling, inducing apoptosis. However, they tested that hypothesis and concluded that the aberrant patterns of methylation are not random (81). Frank Lyko *et al* (2004) showed that the levels of DNA methylation in CLL patients were very heterogeneous (82). High levels of genomic methylation were associated with IgHV unmutated patients and consequently poor-prognosis. A more recent study demonstrated that patients with high methylation index (MI) relative to age matched controls have an increased likelihood of requiring treatment, and low MI was associated with no need of therapy (41).

Posteriorly to those studies, were performed microarray-based studies where it was identified over 100 genes that suffer hypermethylation in CLL compared with normal B cells and some of those genes were preferentially methylated in cases of high or low expression of CD38 (83,84). For example, *NRP2, SFRP2* and *ADAM12* were preferentially methylated in CD38 high cases (poor prognostic), whereas methylation of *DLEU7* was found in CD38 low cases (good prognostic) (83). Other studies showed that some aberrant methylation patterns are only observed on certain CLL subgroup of patients with specific characteristics, supporting the idea that this disease is extremely heterogeneous (85,86). For example, it was also demonstrated that there are some aberrantly methylated targets that are more frequently found in patients with alterations in chromosomes 11, 17 and 19, which confer a bad prognosis (87). In another study, Cahill and Rosenquist (2013) identified a differential methylation pattern that distinguish poor-prognostic IgHV-unmutated CLL patients from favourable prognostic IgHV mutated CLL patients (41). They demonstrated that genes involved in MAPK and NF-kB pathways, which are responsible for cell proliferation and survival, were not methylated in IgHV-unmutated patients compared with mutated ones.

Recently it emerged two new next generation high-resolution methylation profiling techniques called reduced representation bisulfite sequencing (RRBS) and whole-genome bisulfite sequencing (WGBS) (52,87). A WGBS study permitted to conclude that the methylome of cancer cells frequently contains hypomethylated promoters,

comparing to their normal cellular counterparts and it results in increased gene expression (88). This fact is verified in another study, where promoter hypomethylation events showed to be ten times more frequent than hypermethylation events in both IgHV mutated and unmutated CLL patients (87). Furthermore, unmutated CLL patients contain more than four times more hypomethylated promoters, showing a possible role in CLL pathogenesis that requires further research. Some DNA methylation studies display the importance of hypomethylation events for tumorigenesis by contributing to proto-oncogene activation and genomic instability that increase the propensity of genomic alterations (89,90). However, it has become apparent that oncogenes activation by DNA hypomethylation is rather infrequent in CLL, being more frequent tumour suppressor gene inhibition by DNA hypermethylation (52). Though, these results cannot be generalized to all CLL patients because Stach *et al* (2003) demonstrated, that genomic hypomethylation has a high degree of interindividual variation (84). Despite promoter hypomethylation being somehow frequent in CLL patients, its importance to the pathogenesis of the disease is still under investigation, since promoter hypermethylation and consequently gene silencing seems to have more impact in CLL pathogenesis because of its target genes.

Some genome studies were performed proving that *BCL2, MCL1, ODC, ERB-A1* and *TCL1* gene expression are increased in CLL by hypomethylation (Table 2) (91,92). *ODC* plays an important role on cell growth and *ERB-A1* is an oncogene that promote CLL cell survival since their expression is enhanced (93). BCL2 and MCL1 proteins sequester pro-apoptotic members of the BCL2 protein family preventing mitochondrial dysfunction and apoptosis, and in CLL pro- and anti-apoptotic signals seem to converge on MCL1, which suggest its critical role in regulating survival in this disease (94). High levels of BCL2 and MCL1 or failure to decrease MCL1 levels is associated with poor responses to chemotherapy, disease progression, and inferior survival in CLL (95). *TCL1* gene product acts as a coactivator of AKT serine-threonine kinase which translocate to the nucleus and promotes cell survival via NF-kB pathway (96). A study made in transgenic *Eμ-TCL1* mouse model of CLL, generated by overexpression of the *TCL1* oncogene in mouse B-cells, provided strong evidence that aberrant hypomethylation participates in CLL development (97). Mice developed CLL at 18 months of age which allowed researchers to associate hypomethylation of *TCL1* gene with CLL development.

On the other hand, *TWIST-2* is another gene that suffers alteration of its methylation pattern and this methylation appears to be characteristic of CLL (98). This gene product has the function of silencing the *TP53* pro-apoptotic gene but, since *TWIST-2* gene is silenced by hypermethylation, consequent silencing of *TP53* does not take place and the apoptotic process may occur. Therefore, this gene appears to be differentially methylated in CLL patients according to the mutation of IgHV. It was demonstrated to be more frequent in mutated IgHV patients (favourable prognosis) that in unmutated ones, indicating its role in patient's prognosis (41).

Table 2 – Genes with aberrant promoter methylation in CLL.

Gene symbol	Gene name	Function	Methylation prevalence (%)	Ref.
		Hypermethylated genes		
ADAM12	ADAM Metallopeptidase Domain 12	Protease	72	(83)
APC2	Adenomatous Polyposis Coli 2	WNT signaling pathway	77	(83)
BTG4	B-Cell Translocation Gene 4	Cell cycle control (G_1-S)	47	(101)
CALCA	Calcitonin Related Polypeptide Alpha	Calcium metabolism	100	(102)
CD38	cluster of differentiation 38	Cell adhesion	58	(101)
CDH1	Cadherin 1	Cell adhesion	60	(102)
CDH13	cadherin 13	Cell adhesion	70	(103)
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A	Cell cycle control (G_1-S)	20	(102)
CDKN2B	Cyclin Dependent Kinase Inhibitor 2B	Cell cycle control (G_1-S)	50	(102)
RBP1	Aberrant cellular retinol binding protein 1	Retinol transport	28	(104)
CRY1	Cryptochrome Circadian Clock 1	Circadian gene – cell cycle DNA damage response	0-40	(105)
DAPK1	Death Associated Protein Kinase 1	Cell death – tumor suppressor gene	100	(98)
DKK3	Dickkopf-related protein 3	WNT signaling pathway	18	(106)
DLC1	Deleted in liver cancer 1	Putative tumor suppressor gene	69	(83)
DLEU7	Deleted in lymphocytic leukemia 7	Putative tumor suppressor gene	58	(83)
DMRT2	Doublesex and Mab-3 Related Transcription Factor 2	Sex determination	47	(83)
DUOX2	Dual Oxidase 2	Oxidase	37	(83)
CHD1	Chromodomain Helicase DNA Binding Protein 1	Cell-cell adhesion	78	(107)
FOXE1	Forkhead Box E1	Transcription factor	-	(81)
GLRB	Glycine Receptor Beta	Involved in the nervous system functions	-	(81)
GRM7	Glutamate Metabotropic Receptor 7	Inhibit cyclic AMP signaling	-	(81)
HOXA4	Homeobox A4	Homeobox gene	38	(108)
HOXA5	Homeobox A5	Homeobox gene	59	(108)
HOXA6	Homeobox A6	Homeobox gene	34	(108)
HOXC10	Homeobox C10	Homeobox gene	79	(83)
ID4	Inhibitor of DNA Binding 4, HLH Protein	helix-loop-helix transcription factors (tumor suppressor)	100	(109)
PDX1	Insulin promoter factor 1	Transcription factor	-	(81)
KCNK2	Potassium two pore domain channel subfamily K member 2	Ion channel	29	(83)
LEF1	Lymphoid Enhancer Binding Factor 1	Survival/proliferation related gene	-	(85)
LPL	Lipoprotein Lipase	Prognostic marker of CLL	58	(110)
LHX2	LIM Homeobox 2	Homeobox gene	69	(83)
LRP1B	LDL receptor related protein 1B	Low density lipoprotein receptor	56	(83)

Table 2 – Genes with aberrant promoter methylation in CLL (continuation).

NOTCH1	-	Transmembrane receptor	10-15	(111)
NRP2	Neuropilin 2	Neuropilin family receptor	45	(83)
TP53	-	Tumor suppressor gene	10-17	(112)
PAK5	P21 (RAC1) Activated Kinase 5	Involved in the nervous system functions	-	(81)
PCDHGB7	Protocadherin gamma subfamily B, 7	Cell adhesion	100	(83)
POU3F3	POU class 3 homeobox 3	Homeobox gene	77	(83)
RARβ	Retinoic Acid Receptor Beta	Signal transduction	29-35	(103,104)
RLN2	Relaxin 2	Hormone	63	(83)
SF3B1	Splicing Factor 3b Subunit 1	Component of the u2 snRNP sliceosome	5-18	(113)
SFRP1	Secreted frizzled related protein 1	WNT signaling pathway (inhibitor)	-	(106)
SFRP2	Secreted frizzled related protein 2	WNT signaling pathway	69	(83,106)
SFRP4	Secreted frizzled related protein 4	WNT signaling pathway (inhibitor)	-	(106)
SFRP5	Secreted frizzled related protein 5	WNT signaling pathway (inhibitor)	-	(106)
SLIT2	Slit Guidance Ligand 2	Tumor suppressor	80	(114)
TBR1	T-Box, Brain 1	Involved in the nervous system functions	-	(81)
TBX1	T-Box 1	Transcription factor	-	(81)
TBX3	T-Box 3	Transcription factor	-	(81)
TBX18	T-Box 18	Transcription factor	-	(81)
TERT	Telomerase reverse transcriptase	Catalytic subunit of telomerase	47	(115)
TWIST2	Twist family BHLH transcription factor 2	Transcription factor	40	(98)
WIF1	WNT Inhibitory Factor 1	WNT signalling pathway (inhibitor)	12	(116)
ZAP-70	Zeta Chain of T Cell Receptor Associated Protein Kinase 70	Kinase – CLL prognostic gene	50	(117)
Hypomethylated genes				
BCL2	B-cell lymphoma 2	Apoptosis Regulator	95	(118)
THRA	thyroid hormone receptor alpha	Oncogene	-	(81)
MCL1	myeloid cell leukaemia 1	BCL2 Family Apoptosis Regulator	-	(119)
ODC1	Ornithine decarboxylase 1	Cell growth	83	(120)
TCL1A	T-Cell Leukaemia 1A	Activator of NF-kB	-	(121)

Another example of DNA aberrant methylation that appears to be differentially methylated in CLL patients, according to their mutation of IgHV, is the *ZAP-70* gene (99). Mutated IgHV patients have the most methylated promoters, in general, and consequently low expression, which corroborates with the fact that these subgroups of patients have better prognosis (36). On the contrary, its expression is increased in cases of unmethylated *ZAP-70* gene, intensely activating BCR pathway which promotes cell survival and protects cell from apoptosis (100).

HOXA4 gene promoter is also affected by aberrant methylation being silenced by hypermethylation in CLL patients (122). *HOXA4* is part of a family of transcription factors important for cell development and its hypermethylation is most frequent among unmutated IgHV CLL cases (poor prognosis). However, all *HOX* gene clusters seems to be subject of anomalous methylation (53). Two more genes that have hypermethylation of its promoter are *CRY1* and *SLIT2*. *CRY1* is a circadian gene involved in cell cycle and DNA damage response and *SLIT2* is a tumour suppressor gene. Therefore, it can be speculated that deregulation of these genes plays some part in CLL leukemogenesis (114). In another study, Bodoor *et al* (2014) analysed DNA methylation patterns of two cell cycle genes (*TP15* and *TP16*) and four apoptotic genes (*TP14, TP53, DAPK* and *TMS1*) of 70 samples of peripheral blood from leukaemia patients (24 CML, 25 CLL, 12 AML, and 17 ALL) and 24 healthy volunteers (112). In CLL patients the results showed that those genes suffer hypermethylation (25% for *TP14*, 19% for *TP15*, 12% for *TP16*, 17% for *TP53 and* 36% for *DAPK*).

WNT signalling pathway was also studied because it represents a key pathway in B cell development and is intensely activated in CLL (106). According to the study, WNT pathway is activated by hypermethylation of WNT inhibitor genes (*WIF1, DKK3, APC2, SFRP1, SFRP2, SFRP4* and *SFRP5*) and over half of CLL cases showed methylation of at least one inhibitor (123). These results also suggest that these genes play an important role in CLL pathogenesis.

The increase incidence of DNA methylation in CLL cases can be the result of mutations in isocitrate dehydrogenase (IDH) 1 and 2, and ten-eleven translocation 2 (TET2) which demonstrated to inhibit, indirectly or directly, the conversion of 5-mC to 5-hmC causing global accumulation of 5-mC (Figure 4) (124). 5-mC is a methylated form of the DNA base cytosine catalysed by DNA methyltransferase and 5-hmC is the first oxidative product in the active demethylation of 5-mC. The accumulation of 5-mC are associated with the alteration of CpG island methylation leading to deregulation of the expression of various genes (125). As we can see, gene silencing seems to be very frequent in CLL and their consequences need to be studied and used as possible targets to new therapies.

1.3.2. Post-translational deacetylase modification of histones

Histones are important to regulate the accessibility of DNA during cellular processes such as transcription, replication, repair and recombination (126). There are about sixteen types of post-translational histone modifications including methylation, phosphorylation, ADP-ribosylation, ubiquitination and acetylation (127). These modifications control packaging of the DNA that consequently regulate chromatin dynamics and gene

expression. Histone alterations of normal acetylation patterns demonstrate to have a bigger role in ALL and CLL pathogenesis compared to other modifications, being a great target for new therapies (128). Acetylation of the lysine ε-amino group is a dynamic post-translational modification catalysed by histone acetyltransferases (HATs) and its overexpression causes also an increase in gene transcription, since this modification causes neutralization of the positive charges on histones (Figure 11). Histone acetylation functions as a modulator of chromatin structure involved in DNA replication and repair, heterochromatin silencing and gene transcription (129). Disruption of the electrostatic interactions between DNA and histones promote chromatin unfolding and favour the binding of transcription factors into DNA for active gene transcription (130). Histone proteins are not the only ones that suffer acetylation and influence indirectly gene transcription, transcription factors, regulators of DNA repair, recombination and replication, viral proteins and others are also targets of acetylation (129).

However, in ALL and CLL the deacetylation process demonstrates to have more effect on pathogenesis. Histone deacetylase isoenzymes (HDAC) are responsible for deacetylation of histones by removing the acetyl groups from histones, which causes gene silencing and in CLL patients it appears to have a big impact on tumorigenesis (131). Deacetylation process induce the formation of a compacted chromatin structure that is responsible for the repression of transcription (Figure 8). It has been described that patients with carcinoma and leukaemia have elevated levels of HDAC and this abnormal histone deacetylation have been associated with cancer development (132).

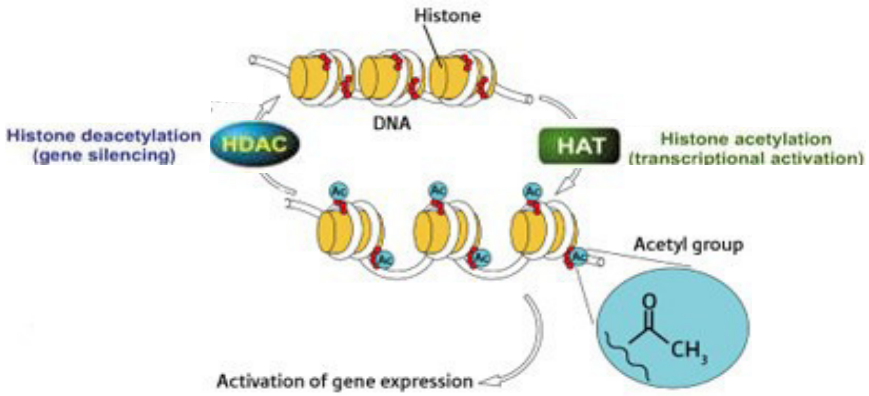

Figure 8 - Acetylation and Deacetylation of histones. HAT enzymes are responsible for the acetylation process and consequently gene expression and the HDAC enzymes are responsible for the reverse process and consequently gene silencing. Histone acetyltransferase, HAT; Histone deacetylase inhibitor, HDAC; Acetyl group, Ac. Adapted from: (133).

Histone deacetylases are chromatin-modulating enzymes that functions by catalysing the removal of negatively charged acetyl groups on specific lysine residues on gene promoters, which triggers the demethylation of lysine 4 on histones. The loss of these epigenetic activating marks promotes chromatin compaction and consequently nucleosome DNA-histone interaction stabilization, preventing the access to transcription factors which leads to gene silencing (134). Up until now, it has been identified 18 different mammalian HDACs that have been divided in four classes.

In haematological malignancies, the increase activity of HDAC may cause transcriptional repression of genes essentially involved in hematopoietic differentiation, contributing to the pathogenesis of several leukaemia, namely APL, AML and CML. Since these processes can be reversed, the development of drug-based treatments for targeting proteins and enzymes involved in the regulation of acetylation/deacetylation can be an innovative therapeutic strategy.

1.3.2.1. Histone deacetylation in ALL

Histone acetylation/deacetylation are involved in the permanent changes of gene expression controlling ALL developmental outcomes (135). Changes in histone deacetylation appears to contribute to carcinogenesis through altered transcriptional regulation of genes involved in processes such as cell cycle regulation, differentiation, apoptosis, cell adhesion and angiogenesis (135).

Increased expression of HDACs, responsible to silence genes, seems to be widespread among cancers and particularly in ALL. Moreno *et al.* identified several increased HDAC genes such as HDAC2, HDAC3, HDAC8, HDAC6, and HDAC7 in ALL samples when compared with normal samples (136). They also show that HDAC6 and HDAC9 were overexpressed in B-cell ALL whereas HDAC1 and HDAC4 were upregulated in T-cell ALL. HDAC2 was also confirmed to be overexpressed in ALL (137). Increased expression of HDAC3, HDAC7 and HDAC9 has been associated with bad prognosis in children ALL because cells were found to display increased HDAC activity and consequently increase gene silencing (136,138)

These types of studies are very recent in ALL and there is few information about which genes are affected by the high levels of HDACs. It is known that HDAC genes are overexpressed and the administration of HDAC inhibitors (HDACi) proved to induce cell death, but the mechanisms behind that are unknown. However, it is known that HDACi promotes the expression of pro-apoptotic genes and inhibit the expression of anti-apoptotic ones. On the contrary, epigenetics in CLL have been studied more intensively with the purpose to understand its importance to pathogenesis and how it can be used to develop more effective treatments.

1.3.2.2. Histone deacetylation in CLL

As mentioned before, HDACs seem to have its expression increased in CLL patients, being responsible for silencing of genes that participate in the development of leukaemia. Thus, it is important to understand and determine which genes are affected to prevent and treat CLL patients. In a study made in 32 CLL patients and 17 normal volunteers, it was demonstrated that most of HDAC isoenzymes appeared to be overexpressed, such as HDAC 1 and 3 from class I; HDAC 6, 7, 9 and 10 from class II and class III; and other deacetylase enzymes such as SIRT1 and 6 (139). The elevated levels were associated with high levels of ZAP-70 indicating that the elevated levels of those isoenzymes are associated with bad prognosis (140).

In CLL, pro-apoptotic genes *BAD, BNIP3L, BNIP3, BIK, BIM, PUMA, MOAP1, AIF* and *SMAC/DIABLO* seem to be silenced by deacetylation and the anti-apoptotic genes *BCL-W, BCL2, BFL-1, XIAP* and *FLIP* seem to

be overexpressed, contributing to cell survival (141). Those results, in association with results obtained from DNA methylation studies, explain why CLL cells appear to be so resistant to apoptotic internal and external signals. Since CLL is nowadays an incurable disease, it is important to try new therapeutic approaches. Another example of a possible therapy, in addition to the DNA hypomethylating agents, is the use of HDACi. It has been studied in CLL cells the use of HDACi and it seems to be a good start for new treatment studies because they allow re-expression of silenced genes involved in cell cycle arrest and apoptosis (53). HDACi cause changes in chromatin compactness which promotes DNA accessibility to the transcription factors and consequently promotes gene transcription. Therefore, HDACi can induce regulation of gene expression, cell cycle arrest in G_1/S or G_2/M, differentiation and apoptosis (141). HDACi action appears to involve the alteration of the acetylation patterns causing the upregulation of *BCL2* family pro-apoptotic genes *(BIM, BMF, BAX and BIK*) and downregulation of anti-apoptotic ones (*BCL2, BCL-X, BCL-W, MCL1, XIAP* and *survivin*) which are critical for apoptosis through the mitochondrial pathway, as observed in two *in vitro* studies (94,141). The expression of *BCL2* family genes showed a decrease in mRNA of anti-apoptotic genes upon administration of HDACi to CLL cells. These results demonstrate that HDACi may be a key to reverse epigenetic alterations that contribute to CLL progression.

In another study, Jordaan *et al* (2014) analysed histone epigenetic alterations of gene promoters in CLL after administration of HDACi (131). It was revealed that CLL cells have higher histone H4 hypo-acetylated gene promoters, but this process is limited to a subset of promoters. Researchers also identified a correlation between acetylation status and some genes such as *BAX, BCL2, CDK4* and *CDKN2B*. *BCL2,* an anti-apoptotic gene, appears to be hyper-acetylated which are in agreement with the DNA methylation studies that indicate an increase of its expression by hypomethylation; *CDK4* gene, that codifies a protein involved in cell division, is also hyper-acetylated which intensifies CDK4 protein function; some tumour suppressor genes such as *CDKN2A* and *CDKN2B* and pro-apoptotic genes like *BAX* seems to be hypo-acetylated, which contributes to CLL tumorigenesis. According to this study, the aim of using inhibitors of HDACs is to increase pro-apoptotic gene transcription such as *BIM* and *PUMA* and to decrease anti-apoptotic genes such as *BCL2* and *XIAP*. However, it was also evident that HDACi do not cause histone hyper-acetylation of all gene promoters. Researchers reported that the exposure of HDACi induce cells to undergo apoptosis, which in turn inhibits the expression of specific anti-apoptotic genes such as *BCL2* and *NFKB1,* resulting in hypo-acetylation of their promoters (131).

In CLL patients, *BCL6* proto-oncogene codify a BTB/POZ-zinc finger transcriptional receptor that is necessary for germinal-centre formation and has been implicated in the pathogenesis of B-cell lymphomas (142). *BCL6* gene expression is regulated by acetylation requiring the recruitment of HDACs to be activated as oncogene. Acetylation prevent *BCL6* ability for recruiting HDACs and consequently inhibits transcription and cellular transformation. Pharmacological inhibition of *BCL6* leads to the accumulation of the inactive acetylated *BCL6* causing cell-cycle arrest and apoptosis in B-cell lymphoma cells. Therefore, the silencing of *BCL6* by acetylation is important to stop the progression of CLL cells being a good target to HDACi (143). Other genes that regulate transcription factors activity like *E2F1, TP53* and *STAT1* are modulated by acetylation and normally becomes hyper-acetylated after administration of HDACi, promoting its expression and function (142). E2F family also

plays an important role in the control of cell cycle and action of tumour suppressor proteins, so its deregulation may contribute to CLL pathogenesis. Other studies using HDAC inhibitors in CLL report that inhibition of HDAC class I but not class II sensitizes tumour necrosis factor-related apoptosis-inducing ligand-induced apoptosis of CLL cells in various cell lines (144,145).

The administration of HDACi seem to be effective for the treatment of ALL and CLL, since they promote the expression of pro-apoptotic genes and consequently induce apoptosis of leukemic cells. Thus, HDACi in combination with other therapies already in use such as chemotherapy, and also combined with other epigenetic treatments, such as DNA hypomethylating agents, may be beneficial and more effective, since it will attack the disease on several fronts.

1.4. Epigenetic therapies

DNA hypomethylating agents and histone deacetylase inhibitors are two categories of epigenetic therapies that may be associated with ALL and CLL since DNA methylation and histone deacetylation are two types of epigenetic modifications most studied in leukaemia. Therefore, it is important to know some examples of therapies that are going to be studied in this project, and understand their mechanism of action, to well understand how and why they can be used to treat ALL and CLL.

1.4.1. DNA hypomethylating agents

As said before, hypermethylation plays a more important role in CLL pathogenesis that hypomethylation because it affects important pro-apoptotic and tumour suppressor genes, inactivating them, promoting cell survival and resistance to apoptotic signals. Therefore, the administration of hypomethylating agents to ALL and CLL patients can be a valuable approach in anticancer therapy leading to reactivation of those silenced genes, antagonizing aberrant tumour proliferation and survival (52). Two examples of hypomethylating agents that are already in use in clinical practice to treat some types of haematological neoplasms that are going to be tested in this project are Azacytidine (5-AC) and Decitabine (DAC). They are being tested alone or in combination with other drugs in ALL and CLL patients but it represents only preliminary phase 1 and 2 studies, some already terminated and others still recruiting patients (146–149).

1.4.1.1. 5-Azacytidine (Vidaza)

5-AC (Figure 9) is an anti-cancer drug characterized as an antimetabolite of cytidine and DNA demethylating agent. It is approved by Food and Drug administration (FDA) and European Medicines Agency (EMA) to treat myelodysplastic syndrome (MDS) and chronic myelomonocytic leukaemia (CMML) (150,151)

Azacytidine is a pyrimidine nucleoside analogue that demethylates or interferes with DNA methylation by inhibiting an enzyme called DNA methyltransferase. This inhibition causes activation of some genes involved in cell proliferation. 5-AC is incorporated into both DNA and RNA leading to inhibition of DNA, RNA and protein synthesis. Upon uptake into cells, 5-AC is phosphorylated three times before incorporated into RNA, causing disruption of nuclear and cytoplasmic RNA metabolism and inhibition of protein synthesis. To be incorporated into DNA, 5-AC is initially phosphorylated two times, then reduced one time and finally phosphorylated one more time, leading to inhibition of DNA synthesis (Figure 10). However, being 5-AC a ribonucleotide, it incorporates into RNA more than into DNA. Azacytidine when incorporated in DNA and RNA, it produces a direct cytotoxic effect on abnormal hematopoietic cells in the bone marrow that causes death of those rapidly dividing cancer cells. 5-AC is most toxic during S-phase of the cell cycle. The normal non-proliferating cells are relatively insensitive to Azacytidine, being not affected in the treatment.

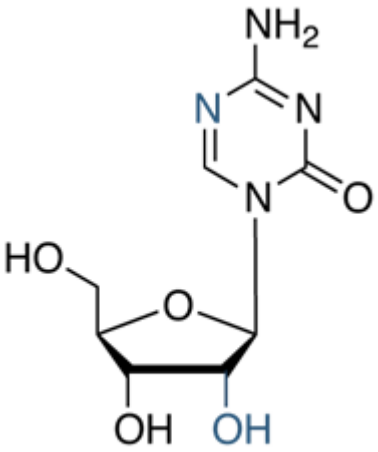

Figure 9 - 5-azacytidine (5-AC) chemical formula.

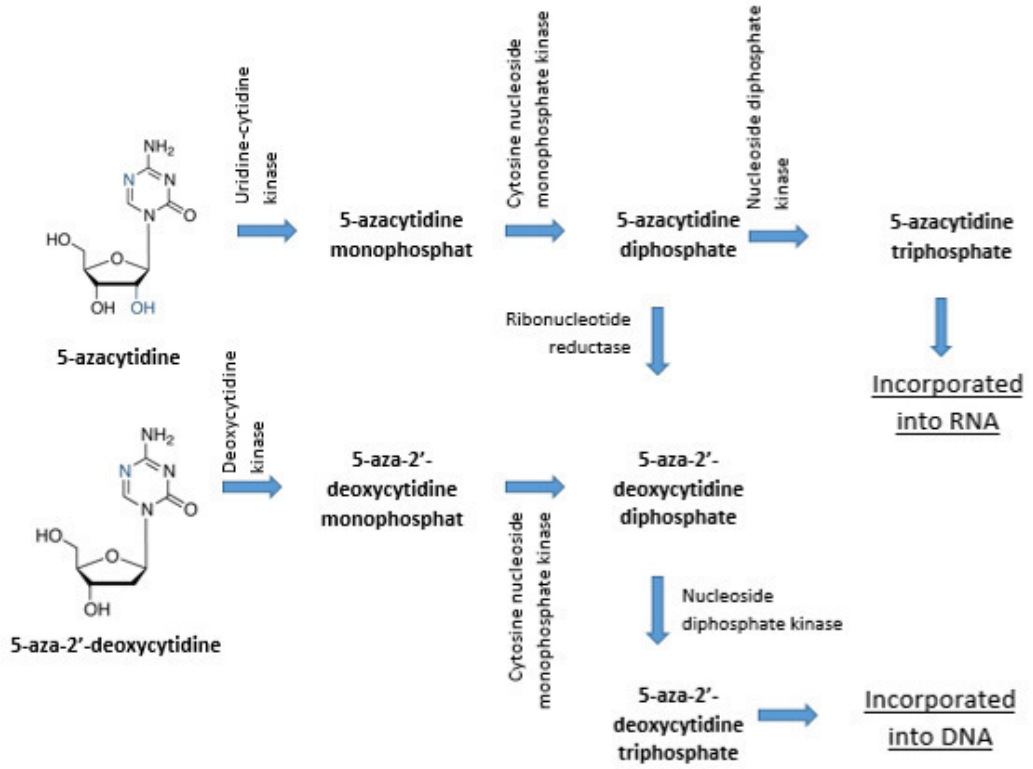

Figure 10 - Mechanism of action of Azacytidine and Decitabine. After incorporation into RNA, 5-AC promotes the disruption of nuclear and cytoplasmic RNA metabolism and inhibition of protein synthesis. 5-AC e DAC both promote inhibition of DNMT1 and DNA synthesis when incorporated into DNA. 5-AC rings bind covalently to DNMT1 forming adducts that are then excised from DNA, ubiquitinylated and degraded by proteasome. This induces the loss of methylation in one of the DNA daughter molecules causing reactivation and re-expression of silenced genes that restore their normal functions.

1.4.1.2. 5-aza-2'-deoxycytidine (Decitabine)

DAC (Figure 11) is an antineoplastic or cytotoxic anti-cancer chemotherapy drug used to treat MDS (152,153). It is classified as an antimetabolite and a DNA demethylation agent. Its mechanism of action is very similar to 5-AC, demethylating or interfering with the methylation of DNA, also producing a direct cytotoxic effect that causes cancer cell death of rapidly dividing cells. This cytotoxicity is due to formation of covalent adducts between DNA methyltransferase and Decitabine.

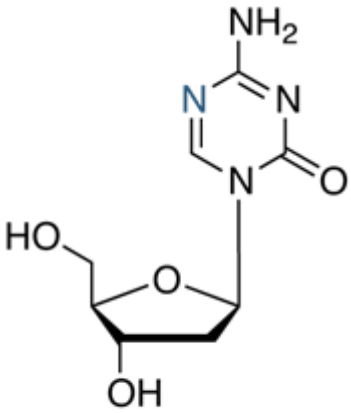

Figure 11 - 5-aza-2'-deoxycytidine (DAC) chemical formula.

DAC is only incorporated into DNA and is more cytotoxic than Azacytidine for cultured cells. DAC is an analogue of 2'-deoxycytidine nucleoside, that needs to be phosphorylated three times for being incorporated into DNA (Figure 10). At low levels, DAC incorporates into DNA of cultured cells, leading to rapid loss of DNMT activity, since these enzymes become irreversibly bound to cytosine residues in DNA. This leads to inhibition of DNA synthesis due to lack of DNMT1 repair (mammalian maintenance methyltransferase) that does not occur for at least two cell cycles. DAC is cell cycle specific acting in S phase, not inhibiting cell progression from G_1 to S phase. The normal non-proliferating cells are relatively insensitive to DAC not being affected in the treatment.

1.4.2. Histone deacetylase inhibitors (HDACi)

HDAC inhibitors were developed with the realization that apart from genetic mutations, the alteration of HDAC enzymes affected the phenotypic and genotypic expression in cells, which in turn lead to disturbed homeostasis and neoplastic growth. Nowadays, despite some HDACi being used for the treatment of some cancers, including some types of leukaemia, none is used to treat ALL and CLL.

However, some phase 1 and small phase 2 studies are in course to determine if some approved HDACi may be administered to CLL patients too. Some examples of HDACi that are being tested are Vorinostat (SAHA), Depsipeptide, Mocetinostat (MGCD0103) and Panobinostat. These compounds have shown promising results in therapy for human lymphoid cancer (145,154–157). Since HDAC inhibitor studies in some leukaemia have promising results, two HDACi, Panobinostat (LBH589) and Vorinostat (SAHA), are going to be tested in this study.

1.4.2.1. Panobinostat (LBH589)

Panobinostat (LBH589) (Figure 12) is a novel broad-spectrum HDACi belonging to the hydroxymate group, that cause an increase of the histone acetylation levels of H3 and H4

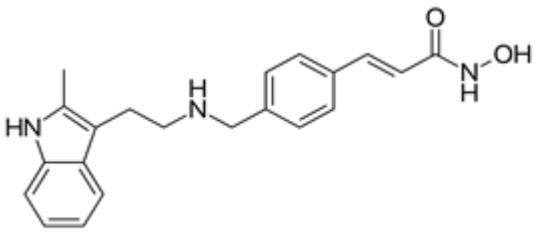

Figure 12 - Panobinostat (LBH589) chemical formula.

histones promoting the transcription of genes. It is administered to treat multiple myeloma in combination with other drugs such as bortezomib and dexamethasone (158). It is also classified as an anti-angiogenic agent by preventing the growth of new blood vessels. Panobinostat inhibits class I (HDACs 1, 2, 3, 8), class II (HDACs 4, 5, 6, 7, 9, 10) and class IV (HDAC 11) proteins promoting accumulation of acetylated histones, inducing cell cycle arrest and/or apoptosis. Inhibition of those proteins may cause reactivation of tumour suppressor genes promoting apoptosis of cancer cells.

1.4.2.2. Vorinostat (SAHA)

Suberoylanilide hydroxamic acid (Figure 13) is a histone deacetylase inhibitor belonging to the hydroxymate group used for the treatment of cutaneous T-cell lymphoma (159). Vorinostat has anti-neoplastic activity inhibiting the histone deacetylase enzymatic activity of class I (HDAC1, HDAC2 and HDAC3) and of class II (HDAC6) (at nanomolar concentrations). However, Vorinostat does not inhibit HDACs belonging to class III. SAHA causes the accumulation of acetylated histones inducing cell cycle arrest at G_1 phase and/or apoptosis of some altered cells in haematological malignancies and solid tumours, using both transcription and transcription-independent mechanisms. The mechanism of the anti-neoplastic effect of SAHA has not been fully characterized but it is known that it binds its phenyl ring to the zinc atom of the catalytic site of the HDAC enzymes. Inhibition of HDAC alters the balance between pro and anti-apoptotic proteins promoting cell death by activation of extrinsic and intrinsic pathways.

Figure 13 - Vorinostat (SAHA) chemical formula.

1.5. Objectives of the study

The present study has two main goals. First is the identification of the therapeutic potential of two DNA methyltransferase inhibitors (Azacytidine and Decitabine) and two histone deacetylase inhibitors (Panobinostat and Vorinostat), administered in single dose, in combination and in daily dose, in ALL and CLL, using two B-ALL cell lineages and CLL primary culture cells. Second is the evaluation of the methylation gene profile in the two B-ALL lineages and CLL samples in order to identify the targeted methylated genes.

2. Material and Methods

2.1. Studies in the two B-ALL cell lines (697 and KOPN8 cells)

2.1.1. Characterization of the cell lines

In this study, it was used two *in vitro* models of human B cell precursor leukaemia, the 697 and KOPN8 cell lines, obtained from Leibniz institute DSMZ-GERMAN Collection of Microorganisms. 697 cells were established

from the bone marrow of a 12-year-old boy with ALL at relapse in 1979 (160). These cells express BCL2, BCL3 and MYC mRNA and have a near diploid karyotype - 46 (45-48)<2n>XY. However, 697 cells have some genetic abnormalities including t(1;19)(q23;p13), del(6)(q21) - and express TCF3-PBX (E2A-PBX) fusion gene. 697 cell line have a significant higher methylation of *TP73, RARB, ESR1, CDKN2A, MGMT, CD44, CADM1, THBS1, CDH13, STK11* and *GATA5* gene promoters, *PAX6* and *WT1* gene promoter are hemi-methylated and *PYCARD* is slightly methylated. KOPN8 was established from the peripheral blood of a 3-month-old girl with B-cell precursor ALL (BCP-ALL) of type B-III in 1977 and were described to carry the t(11;19)(q23;p13) responsible for the MLL-MLLT1 (MLL-ENL) fusion gene (161). This cell line have an hypodiploid karyotype with 4% polyploidy - 45(42-45) <2n> XX, -14, t(8;13)(q24;q21.2), t(11;19)(q23;p13), der(13)t(13;14)(p11;q11). Morphologically, KOPN8 cells are smaller than 697 cells and form clusters in suspension (Figure 14). KOPN8 have a significant higher methylation of *RARB, ESR1, PAX6, CADM1, THBS1, CDH13* and *GATA5* gene promoters, *WT1* gene promoter is hemi-methylated and *CD44* and *STK11* are slightly methylated. KOPN8 cells need more time to proliferate than 697 cells since its doubling time is 48 hours comparing with between 30 to 40 hours for 697.

For this study, the two lineages were suspended in Roswell Park Memorial Institute 1640 (RPMI 1640) medium constituted by 2 mM L-Glutamine, 20 mM of HEPES-Na, 1.5 g/L of NaHCO, 100 U/mL of penicillin, 100 µg/mL of streptomycin with pH 7.4 and enriched with 10% of fetal bovine serum (FBS) (Gibco, Invitrogen). 697 and KOPN8 cells were incubated at 37ºC with 5% CO_2 in a humid ambience.

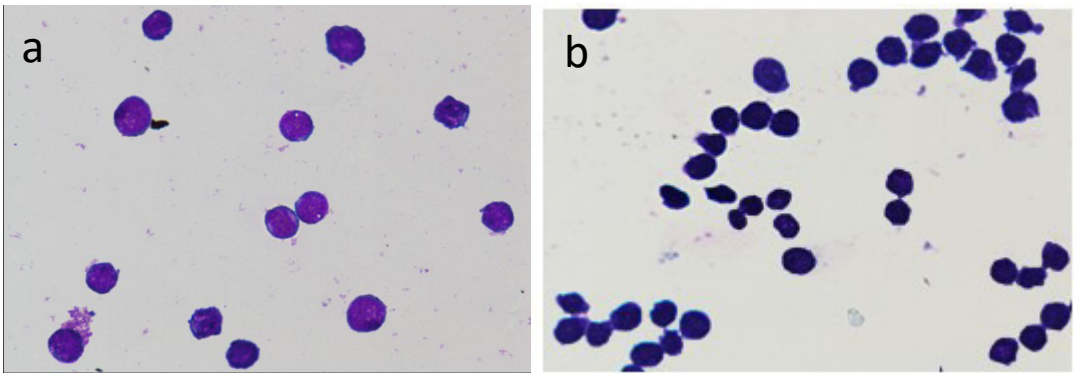

Figure 14 - Morphological aspect of 697 (a) and KOPN8 (b) cell lines, stained with May-Grunewald-Giemsa coloration. Cells are magnified 500x. Cells have a higher nucleus cytoplasmic ratio with little cytoplasm. KOPN8 cells are smaller than 697 cells.

2.1.2. Cell viability and density test

To study cell viability and density, it was used the Trypan blue exclusion test (Thermo Fisher, Grand Island. New York, USA). Trypan blue (0.4% solution) is normally used as a cell stain to study cell viability by colorimetric exclusion test (162). This test is routinely performed for cell count with haemocytometer and is based on the concept that viable cells has an intact cellular membrane and are impermeable to trypan blue, while dead cells are permeable to the dye and acquire the colour blue when observed in the microscope. Trypan blue was added to cell samples in a proportion of 1:1 and the viable/unviable cells were counted using the haemocytometer

(Neubauer chamber). Cell viability was calculated as the number of viable cells divided by the total number of cells within the grids on the haemocytometer and is presented as percentage (%).

2.1.3. Incubation of ALL cell lines with the anticancer drugs in study

697 and KOPN8 cells were incubated for 72h with a density of 0.5 million of cells/mL ($0.5x10^6$ cells/mL) with crescent concentrations of 5-AC (0.5 µM -20 µM), DAC (0.5 µM -15 µM), LBH589 (1 nM - 20 nM) and SAHA (0.1 µM -2.5 µM) in monotherapy. For the combination studies, cells were incubated with 15 µM of 5-AC, 1 µM of DAC, 5 nM of LBH589 and 0.5 µM of SAHA. Daily dose administration studies were performed by administering 5 µM of 5-AC, 1 µM of DAC, 1.67 nM of LBH589 and 2.5 µM of SAHA, for three consecutive days to obtain the IC_{50} dose. 5-AC and DAC were combined with LBH589 and SAHA, simultaneously and with 3-hour of difference between the administrations (5-AC/DAC + LBH589/SAHA; 5-AC/DAC-3h-LBH589/SAHA and LBH589/SAHA-3h-5-AC/DAC).

2.1.4. Evaluation of therapeutic potential of new drugs - quantitative evaluation of cell proliferation and toxicity

2.1.4.1. Fluorometric Microculture Cytotoxicity Assay (FMCA)

The fluorometric microculture cytotoxicity assay (FMCA) is a nonclonogenic microplate-based cell viability assay that measure the cytotoxic and/or cytostatic effect of different compounds *in vitro* (163). FMCA can measure the proliferation activity of cells (total living cells) over a few days (2-4 days) as an advantage over clonogenic assays that can only be tested after 2-3 weeks of agar medium cultures. This assay is based on the hydrolysis of fluorescein diacetate (FD) probe by esterases in cells with intact plasma membranes. Despite of providing similar results from colorimetric assays, FMCA assay is more sensitive to measurements.

Prior to this test, as said in 2.1.3, ALL 697 and KOPN8 cells were incubated for 72h and exposed to the anticancer drugs in monotherapy, as single dose and in daily dose administration, and in drug combination, simultaneously and administrated with 3h intervals. Next, the plates were centrifuged for 5 minutes at 200xG to remove the supernatant, then it was added PBS and centrifuged again to remove the remained medium. The final step is the addition of FD solution to the plate which is composed by FD stock (FD (Sigma) dissolved in dimethyl sulfoxide (DMSO) and FD buffer (NaCl 125, HEPES and Millipore water with adjusted pH of 7.4). This step is done immediately before addition to the plate to avoid precipitation. Then, the plates were incubated at 37ºC for 40 min and the fluorescence was read at 485/520 nm, in the spectrophotometer (Synergy™ HT Multi-Mode Microplate Reader, Biotek Instruments) with a sensitivity of 35%. The data is normalized to control and presented as percentage of cell viability.

2.1.5. Apoptosis detection, cell type differentiation and Intracellular labelling by flow cytometry

Flow cytometry provides rapid analysis of multiple characteristics of single cells and the information obtained is both qualitative and quantitative (164). Flow cytometry measures optical and fluorescence characteristics of single cells and provide information of physical properties like size and complexity, that can characterize certain cell populations. Fluorescent dyes can bind or intercalate with different cellular components and can be conjugated with antibodies to bind to specific proteins on cell membrane or even inside the cell. When the labelled cells pass by the light source it excites the fluorescent molecules to a higher energy state and upon returning to its resting state, the fluorochromes emit light energy with higher wavelengths. The recurrence to monoclonal antibodies with the ability to bind to surface proteins permits cell flow cytometry analysis (165).

2.1.5.1. Cell death analysis (Annexin V/Propidium Iodide)

Annexin V is a cellular protein that can bind to a specific type of phospholipid, named phosphatidylserine (PS), located on the cytoplasmic internal layer of the cell membrane in normal viable cells (166). However, in apoptotic cells, PS is translocated from the inner layer of the cell membrane to the outer layer, exposing PS to labelled annexin V. This translocation is responsible to differentiate viable cells from apoptotic ones by flow cytometry. Propidium Iodide (PI) is a membrane impermeant dye that binds to double stranded DNA by intercalating between base pairs with little or no sequence preference and emits fluorescence. PI is used to identify necrotic cells or late apoptotic cells because they lose their membrane integrity, permitting PI to enter and bind to DNA. When combined with annexin V it is possible to distinguish viable cells (negative to both annexin V and PI), cells on initial apoptosis (positive to annexin V and negative to PI), cells in late apoptosis/necrosis (positive to both annexin V and PI) and cells in necrosis (negative to annexin V and positive to PI).

697 and KOPN8 cells were labelled with annexin V (BioLegend, San Diego, California, USA) and PI (Immunostep, Salamanca, Spain). The protocol involves addiction of Phosphate-buffered saline (PBS) to the cell sample and centrifugation at 300xG for 5 min. Then, 2.5 µL annexin V, 2 µL PI and 100 µL of cold binding buffer for annexin V were added to the sample and incubated for 15 minutes at dark. The final step is the addition of 300 µL of binding buffer to enforce annexin V ligation to PS. Cells were analysed on a FACScalibur flow cytometer equipped with an argon laser. The excitation wavelengths were 525 nm for the annexin V-FITC and of 650 nm for the PI. Through the CellQuest ™ program, 10000 cells were acquired and the results obtained were later analysed using the Paint-a-GateTM program, being an example presented in Figure 15. The results are presented in percentage of each of the cell populations identified based on positivity and/or negativity for double staining with Annexin V and/or PI.

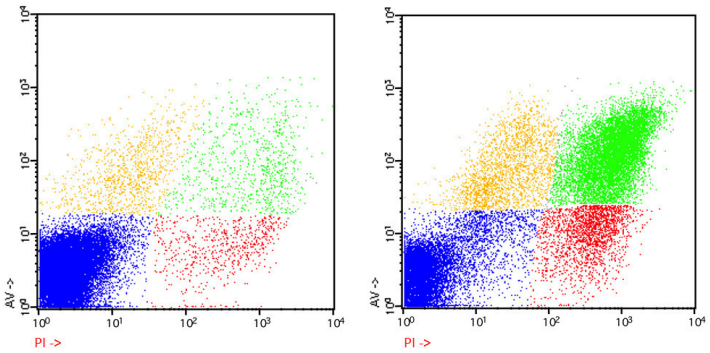

Figure 15 - Example of a dot plot of cell death analysis (AV/PI) obtained by flow cytometry. Results are presented in percentage based of positivity and/or negativity for double staining with Annexin V and/or PI of cell populations. Left – control sample; Results represent number of cells. Right – Cells treated with epidrugs. Yellow- Cell population positive for Annexin V (apoptosis); Green- Cell population positive for Annexin V and Propidium Iodide (Late apoptosis/necrosis); Blue- Cell population negative for Annexin V and Propidium Iodide (live cells); Red- Cell population positive for Propidium iodide (necrosis); Annexin V, AV; Propidium Iodide, PI.

2.1.5.2. Cell cycle analysis

Cell cycle analysis allows to study the distribution of the cells trough the cell cycle phases based on DNA content. DNA has the ability to bind to a variety of DNA binding dies such as PI (167). This die binds in stoichiometric proportion to the amount of DNA present in the cell, so if cells are in S phase they will have more DNA and consequently more signal than cells in G_1 phase, for example. For dies to enter in the cell and bind to DNA, cells need to be permeabilized by adding for example ethanol, to the samples.

Initially, cells were washed by centrifugation at 300xG for 5 minutes, then while vortexing it was added ethanol at 70% to ensure fixation of all cells and minimize clumping. Cells were incubated for 30 minutes at 4°C and then added PBS and centrifuged for 5 minutes at 300xG to remove the medium. Finally, it was added 300 µL of PI and RNAse solution (Immunostep, Salamanca, Spain) to ensure that only DNA, not RNA, was stained and incubated for at least 15 minutes. Finally, samples were stored at 4ºC until reading. An example of the results obtained in this technique are showed in Figure 16.

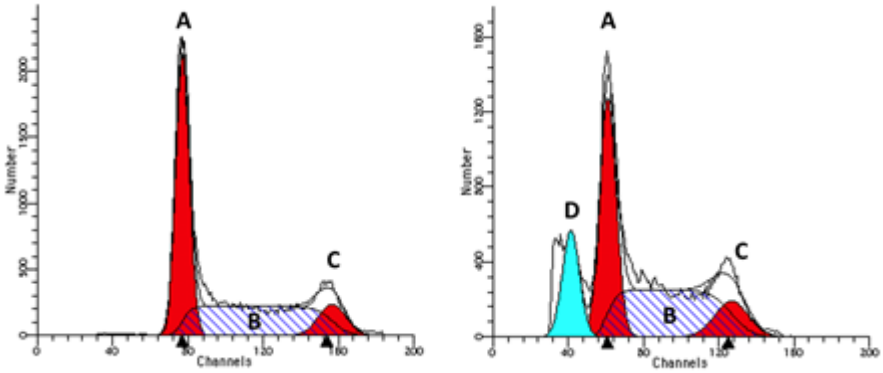

Figure 16 - Example of a dot plot of cell cycle analysis obtained by flow cytometry. Results represent number of cells. Left – control sample; Right – Cells treated with epidrugs; A-G_1 peak (G_0/G_1 phase); B- S peak (S phase); C- G_2 peak (G_2/M phase); D-Sub G_0/G_1 peak.

2.1.5.3. Measurement of 5-mC by flow cytometry

The expression of 5-mC was studied in ALL cell lines which was determined by intracellular labelling with antibodies and detected by flow cytometry. As mentioned before, 5-mC is a methylated form of cytosine catalysed by DNA methyltransferase. Therefore, 5-mC detection can give information about the methylation levels of cells in different conditions (168). Data is given in mean fluorescence intensity (MFI) and the obtained results are exemplified in Figure 17, corresponding to a control sample (red) and cells treated with epidrugs (blue).

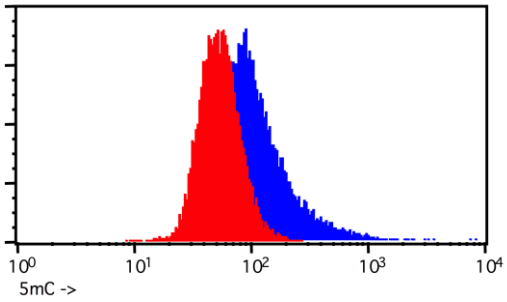

Figure 17- Example of a dot plot for the detection of 5-mC obtained by flow cytometry. Data is given in mean fluorescence intensity (MFI). Red- Control sample; Blue- Cells treated with epidrugs.

Cells were centrifuged at 300xG for 5 minutes after addition of PBS, to remove the medium. Then, it was added solution A (Immunostep, Salamanca, Spain), a fixation solution, and cells were incubated 15 min in dark. Next, cells were washed and centrifuged at 300xG for 5 minutes. After that, it was added solution B (Immunostep, Salamanca, Spain), a permeant solution and the primary antibody of anti-5-mC and cells were incubated for another 15 minutes in a dark environment. Cells were washed again, then it was added the secondary antibody and cells were incubated 30 minutes in dark. Finally, cells were washed, resuspended in PBS and stored at 4°C.

2.1.6. Morphology

The morphologic aspects of 697 and KOPN8 cells induced by the studied drugs were evaluated by the observation of smears on optical microscope. Thus, cells after 72-hour incubation with 5-AC, DAC, LBH589 and SAHA, in monotherapy and combination, were washed and were suspended in FBS. 10µL of cells were used to make smears that were stained using the May-Grunewald-Giemsa protocol (Sigma-Aldrich, St Louis, MO). The smear was covered with May-Grunewald coloration for 3 minutes. Then added MilliQ water for 1 minute. This mixture was decanted and the smear was stained with Giemsa solution for 15 min. The final step is washing the smear under current water and dry in a vertical position. The cell morphology was analysed under the optical microscope Nikon Eclipse 80i, coupled with a digital camera, allowing the recording and processing of imagens using the Nikon ACT-1 program.

2.1.7. Assessment of methylation patters of DNA

2.1.7.1. DNA extraction by quick mini prep salting out

For the study of gene methylation, DNA obtained from ALL cell lines was extracted using salting out DNA extraction protocol. Initially it was added 244 µL of ddH_2O, 80 µL of proteinase K buffer (5x), 40 µL of sodium dodecyl sulphate (SDS) at 10% and 6 µL of proteinase K (50 mg/ml). The solution is homogenised, vortexed and incubated at 55°C for 10 min. Next, samples were placed on ice for 1 min and added 120 µL of NaCl 6M. Cells were centrifuged for 5 min at 16000xG. The supernatant was transferred for another tube, it was added 1 mL of cold absolute ethanol (100%) and samples were centrifuged for 1 min at 16000xG. In this step, DNA is precipitated and it is possible to visualize. The final step is the removal of the ethanol added in the previous step and, after being dry of ethanol, DNA is suspended in ddH_2O and stored at -20°C. DNA was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

2.1.7.2. MS-MLPA (Methylation-specific multiplex ligation-dependent probe amplification)

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) is a semi-quantitative technique used for methylation profiling and nowadays widely used for the detection of epigenetic alterations (169). MS-MLPA is a variant of the MLPA method where copy number detection is combined with the use of a methylation-sensitive restriction enzyme. The protocol is very similar to MLPA, except in MS-MLPA it generates two samples for two different purposes. The protocol is based in five steps: DNA denaturation, hybridisation, ligation/ligation-digestion reaction and PCR.

First DNA was denatured in the thermocycler (5 min at 98°C and then samples were cooled to 25°C before removed from the thermocycler) where the fragments are denatured to single strands of DNA. Next, the samples were hybridised with MLPA probes, where the hybridisation master mix (MLPA buffer and probemix) were added to the samples, still on thermocycler (incubated for 1 min at 95°C and then 16-20 hours at 60°C), allowing the probes to bind to the DNA previously denatured. Then the thermocycler was paused at 20°C, the tubes were removed from the thermocycler and was added ligase buffer A to every tube. In this step samples are duplicated for methylation and copy number studies, being placed again in the thermocycler at 48°C. Thereafter, Ligase-65 master mix (dH_2O, ligase buffer B and ligase-65 enzyme) was added to the hybridised DNA tubes where the undigested samples were used for copy number detection by standard MLPA reaction and Ligase-Digestion master mix (dH_2O, ligase buffer B, ligase-65 enzyme and HhaI enzyme) was added to the hybridised DNA tubes, endonucleases forming digested fragments that are used for methylation detection. Both tubes were incubated for 30 min at 48°C (for ligation and HhaI digestion) and 5 min at 98°C for heat inactivation of the enzymes. On this step occurs the ligation and digestion of the fragments with methylated-sensitive endonucleases. The final step is the PCR where the undigested fragments, still bonded to the probes, are exponentially amplified. Polymerase master mix (dH_2O, SALSA PCR primer mix and SALSA polymerase) was added to each tube, at room temperature, and then tubes are placed in the thermocycler for 35 PCR cycles (30 seconds at 95°C, 30 seconds at

60°C, 60 seconds at 72°C). The process ends with 20-minute incubation at 72°C and then cooled to 15°C. Next, samples were denatured for 2 minutes at 80°C in the thermocycler and then sequenced. After hybridization, the digestion of the hybrid fragments by endonucleases occurs only on unmethylated fragments not allowing them to be amplified during the PCR step and hence will not produce a signal during detection. On the contrary, if the fragments are methylated they become protected against endonuclease digestion, being amplified during PCR and hence generate a signal. All reagents were obtained from MRC-Holland, Amsterdam, Netherlands. All reactions were carried out in a thermocycler equipped with a heat lid (ABI 2720, Applied Biosystems). The fluorescently labelled PCR products were separated by capillary electrophoresis (ABI-PRISM 3130 sequencer, Applied Biosystems) and analysed by Coffalyser.Net software.

The selected assay (ME002-C1, MRC-Holland, Amsterdam, Netherlands) evaluates 25 tumour suppressor genes (*TP73, MSH6, VHL, RARB, ESR1, CDKN2A, PAX5, KLLN, MGMT, PAX6, WT1, CD44, GSTP1, CADM1, CHFR, BRCA2, RB1, THBS1, TSC2, PYCARD, CDH13, TP53, BRCA1, STK11* and *GATA5*). Data is obtained in percentage (%) of methylation and gain (>1.2) or losses (<0.8) of gene expression. In methylation analysis, samples with a methylation level >15% were considered as methylated, and different ranges of methylation were determined: demethylation (0–15%), partial methylation (16–50%), and extensive methylation (>50%). In the copy numbers analysis, a mean value 0.20 lower than control samples were defined as deletion (loss of copy number) and a mean value 0.20 higher than control samples was defined as amplification (gain of copy number). Results are obtained in values and graphs (Figure 18). For this project only methylation data was used.

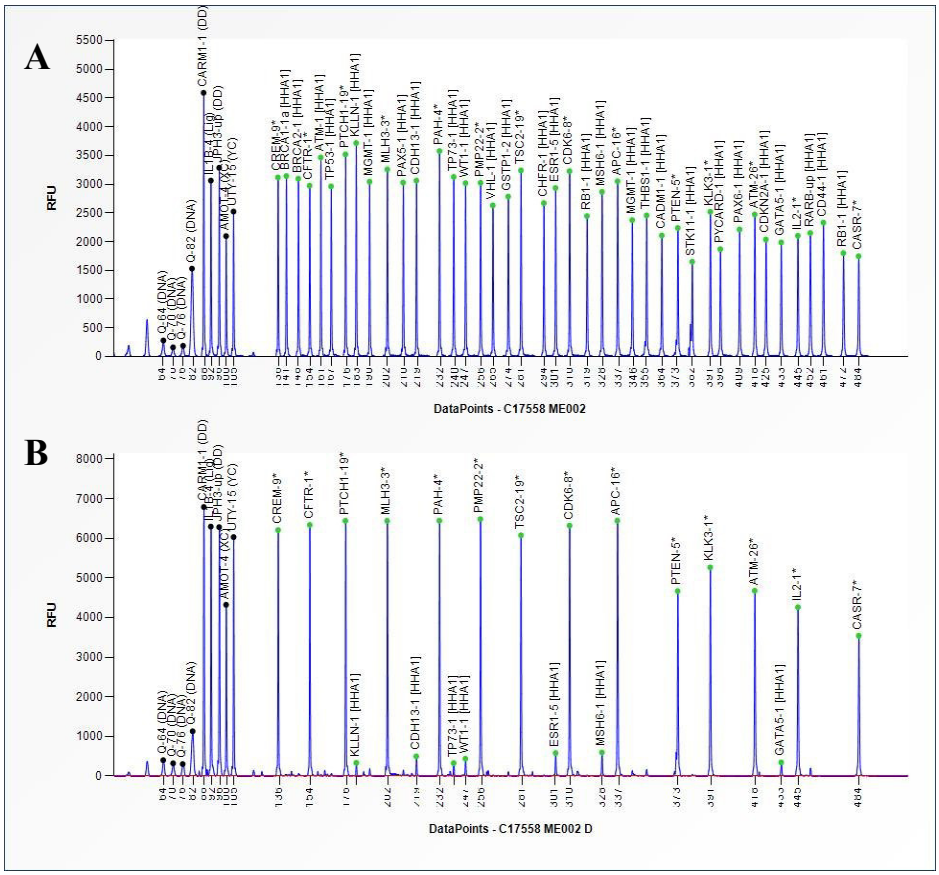

Figure 18 – **Representation of an electropherogram obtained from MS-MLPA technique**. Data is obtained for copy number variations (CNV - A) and methylation pattern (B). This data corresponds to a control.

2.2.Studies in the CLL samples (patients)

2.2.1. Ethical statement

All research procedures were approved by the Ethics Committee of Medicine Faculty of University of Coimbra (Coimbra, Portugal). The peripheral blood samples were obtained after patients provided their informed consent in accordance with the Helsinki declaration. Peripheral blood samples were collected from patients according to Institutional Review Board–approved protocols, fulfilling diagnostic and immunophenotypic criteria for B-cell CLL.

2.2.2. Study population

The present study involved 31 individuals, 21 diagnosed with CLL and 10 non-neoplastic controls, obtained from Centro Hospitalar e Universitário de Coimbra (CHUC) and Hospital Distrital da Figueira da Foz (HDFF). Patients were grouped according to their clinical and demographic features (Table 3).

Table 3- Characteristics of the population in study.

Characteristics	Controls (n=10)	CLL patients (n=20)
Age (years)	66.4 (57-80)	70 (50-87)
Gender		
Male	7	16
Female	3	5
Treatment		
Treated	-	15
Not Treated	-	6
Disease progression	-	4
Rai staging system		
Low risk	-	10
Intermediate risk	-	5
High risk	-	6

2.2.3. Sample preparation (Primary cultures)

2.2.3.1. Mononuclear cells isolation from peripheral blood

In this study, all the blood samples used in this step are collected in heparin or citrate tubes. Peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll Paque density gradient centrifugation protocol (GE Healthcare, Little Chalfont, United Kingdom). Blood was layered on the top of the Ficoll solution, at room

temperature, and was centrifuged at 200xG for 30-40 min. Ficoll at density of 1.073 g/ml promotes the formation of layers, after centrifugation, by differential migration of blood cell types according to its density (Figure 19) (170). The mononuclear cells can be seen in the interface between the Ficoll and plasma layer, since they have intermediate density. Mononuclear cells are then recovered from the interface, centrifuged again and resuspended in RPMI 1640 medium, constituted by 2 mM L-Glutamine, 20 mM of HEPES-Na, 1.5 g/L of NaHCO, 100 U/mL of penicillin, 100 µg/mL of streptomycin with pH 7.4 and enriched with 20% of FBS (Gibco, Invitrogen) to posterior incubate with the drugs in use. The remaining PBMCs not used for viability studies were cryopreserved with RPMI 1640 medium, supplemented with 20% FSB and 10% DMSO; Sigma-Aldrich, St Louis, MO) at -80°C.

Figure 19 – Result obtained after Ficoll protocol. Left – schematic representation of the layers formed after centrifugation. Right- real blood sample layered after centrifugation with Ficoll; Rectangle - location of the mononuclear cells.

2.2.3.2. Incubation of mononuclear CLL cells with the anticancer drugs in study

Mononuclear cells obtained from CLL patients were incubated for 48h at an initial density of 1 million of cells/mL (1x10^6 cells/ml). Cells were incubated with crescent concentrations of 5-AC (10 µM - 50 µM), DAC (10 µM - 50 µM), LBH589 (15 nM - 100 nM) and SAHA (1 µM -7.5 µM), in monotherapy, as single dose or in daily dose administration (single doses for 2 days - 15 µM of 5-AC, 15 µM of DAC, 25 nM of LBH589 and 1.25 µM of SAHA), and in combination (30 µM of 5-AC, 30 µM of DAC, 15 nM of LBH589 and 1 µM of SAHA). 5-AC and DAC were combined with LBH589 and SAHA in simultaneous administration (5-AC+LBH589; 5-AC+SAHA; DAC+LBH589; DAC+SAHA).

2.2.4. Fluorometric Microculture Cytotoxicity Assay (FMCA)

Prior to this test, as mentioned in 2.2.3.2, CLL cells were incubated only for 48h and exposed to anticancer drugs in conditions referred previously. Cell viability was determined according to the protocol described in section 2.1.4.1. The data is normalized to control and presented as percentage of cell viability.

2.2.5. Apoptosis detection and cell type differentiation by flow cytometry

2.2.5.1. Cell death evaluation (Annexin V/ $CD5^+$ and $CD19^+$)

CD5 and CD19 are proteins expressed on the surface of T and B cells, respectively, that can be used to differentiate those two types of cells by flow cytometry (171). Despite CLL cells being B-cells (expressing CD19) they also express CD5 proteins characteristic of T-cells. This fact permits to distinguish CLL cells from normal B cells using antibodies and flow cytometry. When combined with annexin V is possible to distinguish which type of cell suffered apoptosis.

For CLL samples, mononuclear cells were labelled with CD5 and CD19 antibodies and also annexin V. The protocol involved washing the cells with PBS and centrifugation at 300xG for 5 min. Then the CD5 and CD19 antibodies (BD Biosystems) were added to the samples and they were incubated for 15 minutes in the dark. Then the samples were washed and annexin V-FITC was added to the sample in combination with the ligation buffer for annexin V and incubated for another 15 minutes in the dark. The final step was the addition of more ligation buffer to enforce annexin V. CD5 and CD19 antibodies were used to identify normal B cells (CD5-/CD19+), neoplastic B cells (CD5+/CD19+), normal T cells (CD5+/CD19-) and other mononuclear cells (CD5-/CD19-). An example of data obtained from cell death evaluation is presented in Figure 20.

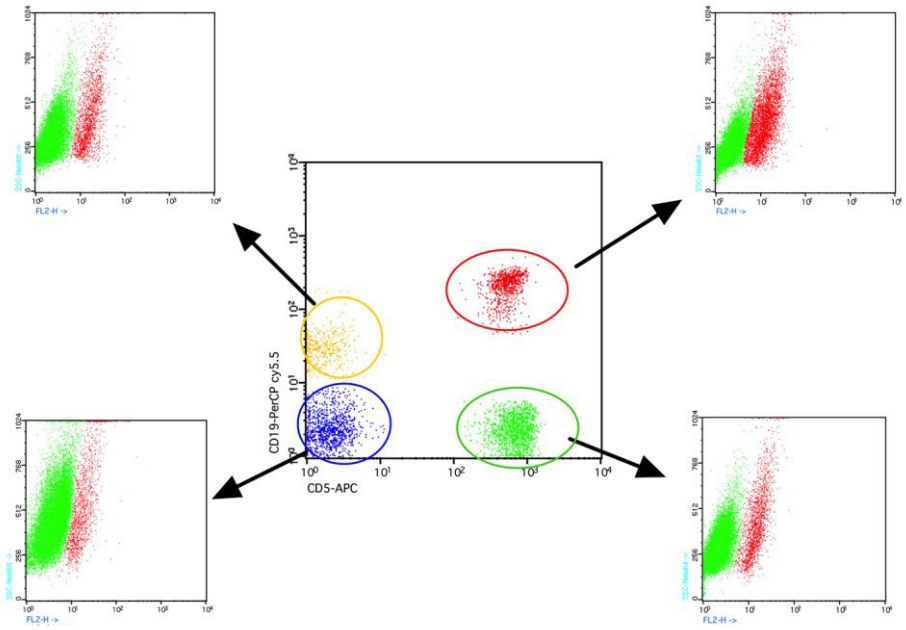

Figure 20 - Example of a dot plot of cell death analysis (AV/CD5/CD19) obtained by flow cytometry. Results are presented in percentage based of positivity and/or negativity for triple staining with Annexin V and/or CD5 and CD19 antibodies of cell populations. Yellow - $CD19^+$ cell population (Normal B lymphocytes); Red - $CD19^+/CD5^+$ cell population (neoplastic B lymphocytes); Blue - $CD19^-/CD5^-$ cell population (other mononuclear cells); Green – $CD5^+$ cell population (normal T lymphocytes).

2.2.5.2. Cell cycle evaluation

To analyse cell cycle distribution of CLL cells incubated in the absence and presence of 5-AC, DAC, LBH589 and SAHA, the procedure described in section 2.1.5.2 was applied. Results are similar to the ones represented in Figure 16.

2.2.6. Assessment of methylation patterns of DNA

2.2.6.1. DNA extraction by quick mini prep salting out

For the study of methylation, DNA from CLL cells was extracted using *salting out* DNA extraction protocol, described in section 2.1.7.1.

2.2.6.2. MS-MLPA (Methylation-specific multiplex ligation-dependent probe amplification)

To analyse the methylation patterns of CLL cells, the procedure described in section 2.1.7.2 was applied.

2.3. Data analysis, statistics

The evaluation of the differences between monotherapy doses, combination schemes, cell cycle and cell death data, as well as intracellular labelling (5-mC detection), on 697 and KOPN8 cell lines, were determined by applying the nonparametric ANOVA Kruskal-Wallis test (Dunn's multiple comparisons test). To determinate the respective IC_{50} of the various compounds in 697 and KOPN8 cell lines, was applied non-linear logistic regression to the 72-hour data. MS-MLPA obtained data were analysed using chi-squared (X^2) test. The probability value of $p<0.05$ was considered statistically significant. To CLL cells results were analysed by applying the same statistical tests referred before.

3 Results

3.1. Evaluation of the therapeutic potential of the epidrugs in ALL cell lines

DNA methylation and histone deacetylation studies in ALL and CLL show that some genes suffer alteration on its expression as mentioned before. That seems to be important for the pathogenesis of those cancers, especially hypermethylation and deacetylation of tumour suppressor genes. Therefore, a new therapeutic approach with the administration of hypomethylating agents and histone deacetylase inhibitors may represent a turning point in ALL and CLL patient survival.

3.1.1. The effect on cell viability of DNA hypomethylating agents (Azacytidine and Decitabine) in 697 and KOPN8 cell lines

In order to determine de therapeutic potential of the DNA hypomethylating agents (Azacytidine and Decitabine), the cytotoxic effect was assessed by FMCA technique as described in Methods. To this end, cells were incubated in the absence (control) and presence of crescent concentrations of 5-AC (0.5 µM-20 µM) and DAC (0.5 µM - 15 µM), for different incubation times (24h, 48h and 72h), with results represented in Figure 21.

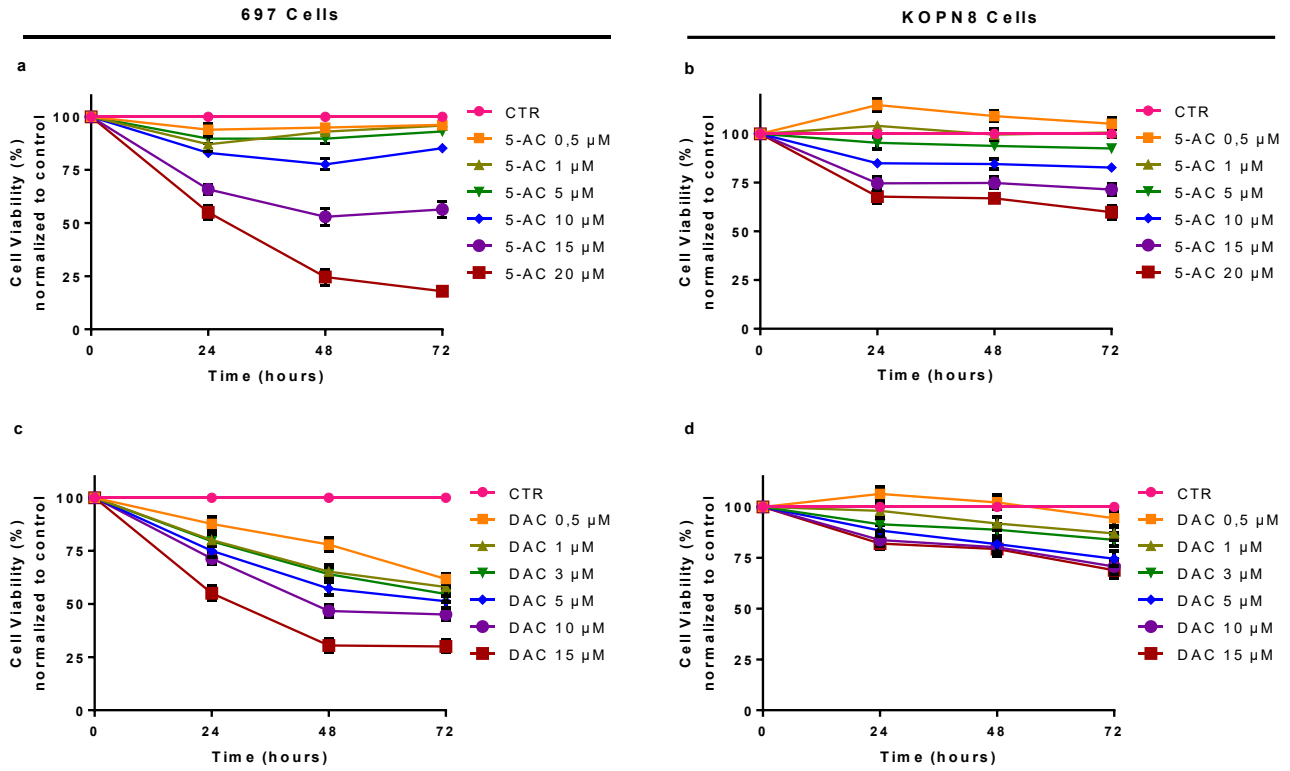

Figure 21 - Dose response curves of 5-AC and DAC administered in monotherapy in 697 and KOPN8 cell lines. 697 and KOPN8 cells were incubated for 72h, in the absence (Control) and presence of different concentrations of Azacytidine (a/b) and Decitabine (c/d) in monotherapy, as indicated in figure. Dose response curves were established by FMCA method each 24h, as described in Methods. Results are expressed in percentage (%) normalized to control. Data are expressed as mean ± SEM obtained from at least 8 independent experiments. Control, CTR; Azacytidine, 5-AC; Decitabine, DAC.

As observed in Figure 21, both DNA methyltransferase inhibitors (5-AC and DAC) demonstrated to have effect on cell viability dependent on dose, incubation time and cell type. 5-AC and DAC demonstrated to have effect on cell viability right after 24h of incubation, intensifying their effect in the next 2 days of incubation. In 697 cell line, after 72h of incubation, doses higher than 10 µM of 5-AC, demonstrated to decrease significantly cell viability ($p<0.05$). As for DAC, doses higher than 3 µM demonstrated to decrease significantly cell viability ($p<0.05$). KOPN8 cell line data, after 72h of incubation, showed that only doses higher than 10 µM of 5-AC and doses higher than 5 µM of DAC decrease significantly cell viability, $p<0.05$ and $p<0.001$, respectively. The half maximal inhibitory concentration (IC_{50}) after 72h of exposure was calculated with recurrence to nonlinear progression, being approximately, 19 µM for 5-AC and 4 µM for DAC in 697 cell line and 38 µM for 5-AC and 23 µM for DAC in KOPN8 cell line. The IC_{50} of these two DNA hypomethylating agents was not reached in KOPN8

cells with tested concentrations (Figure 21 b and d). However, highest doses were not tested since they are not viable on patients and because of the intensification of side effects. In sum, it can be said that 697 cells demonstrated to be more sensitive than KOPN8, to the administration of 5-AC and DAC.

3.1.2. The effect on cell viability of histone deacetylase inhibitors (Panobinostat and Vorinostat) in 697 and KOPN8 cell lines

The effect of the histone deacetylase inhibitors (Panobinostat and Vorinostat) was also determined by FMCA technique as described in Methods. Cells were incubated in the absence (control) and presence of LBH589 (1 nM – 20 nM) and SAHA (0.1 µM - 2.5 µM), during 72h, being the effect presented in Figure 22.

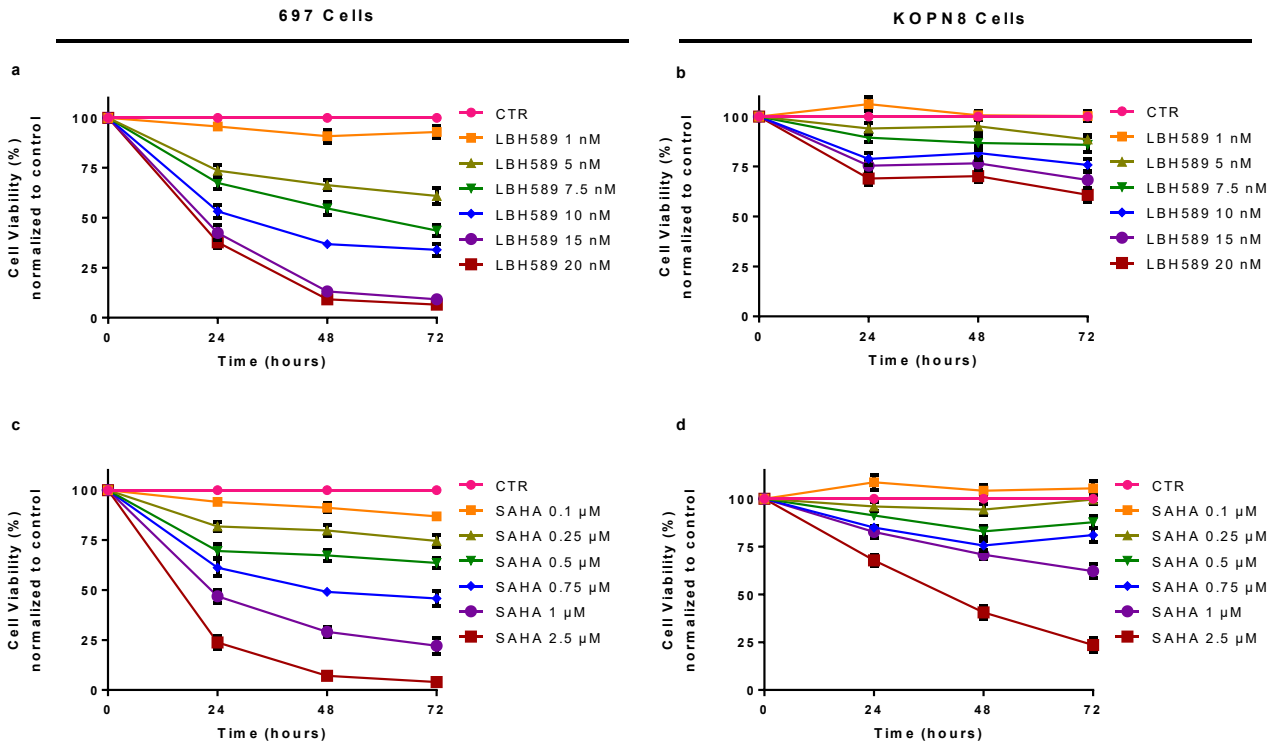

Figure 22 - Dose response curves of LBH589 and SAHA administered in monotherapy in 697 and KOPN8 cell lines. 697 and KOPN8 cells were incubated for 72h, in the absence (control) and presence of increasing concentrations of Panobinostat (a/b) and Vorinostat (c/d) in monotherapy, as indicated in figure. Dose response curves were established by FMCA method each 24h, as described in Methods. Results are expressed in percentage (%) normalized to control. Data are expressed as mean ± SEM obtained from at least 8 independent experiments. CTR correspond to cells with no treatment. Control, CTR; Panobinostat, LBH589; Vorinostat, SAHA

The effect of both HDACi, LBH589 and SAHA, on cell viability was dependent on the dose, incubation time and cell line. As seen with DNA hypomethylating agents, 697 cell line also demonstrated to be more sensitive to LBH589 and SAHA than KOPN8 cells. Panobinostat and Vorinostat demonstrated to have effect on cell viability right after 24h of incubation, being its effect intensified in the next 2 days of incubation, in highest concentrations and mostly on 697 cells. For lowest concentrations, the effect was reached at 24h and maintained slightly constant through the next 48h, with some oscillations. IC_{50} was calculated after 72h of incubation, with recurrence to

nonlinear progression. The results were approximately 5 nM to LBH589 and 0.6 µM to SAHA, for 697 cell line, and 34 nM to LBH589 and 1.9 µM to SAHA, for KOPN8 cell line. KOPN8 IC_{50} was also not reached with tested concentrations for Panobinostat, only for Vorinostat, in a dose between 1 – 2.5 µM (Figure 22 b and d). Only doses higher than 7.5 nM of LBH589 and doses higher than 0.5 µM of SAHA demonstrated to reduce significantly cell viability of 697 cells, $p<0.05$ for both drugs. However, the significant reduction of cell viability for KOPN8 cell line, was only obtained with doses higher than 10 nM for Panobinostat ($p<0.01$) and doses higher than 1 µM for Vorinostat ($p<0.01$).

3.1.3. The effect on cell viability of the combination of DNA hypomethylating agents and histone deacetylase inhibitors and daily dose administration in 697 and KOPN8 cell lines

One of the problems associated with many of the therapeutics used in the treatment of cancer is the development of numerous side effects. Therefore, the combination of therapeutics and daily dose administration permit the achievement of the same therapeutic effect with lowest doses, compared to those used in monotherapy, providing a better response with lower toxicity and consequently, less side effects. Thus, the effect of combination and daily dose administration were determined by FMCA technique, as described in Methods, with the purpose of evaluating their effect on cell viability when administrated simultaneously, with 3-hour delay and daily administration (3 consecutive days).

Cells were incubated in the absence (control) and presence of 5-AC (15 µM), DAC (1 µM), LBH589 (5 nM) and SAHA (0.5 µM), for different incubation times (24h, 48h and 72h) and different therapeutic schemes. The effect of combinations is represented in Figures 23 and 24. For daily dose administration, cells were incubated with 5-AC (5 µM), DAC (1 µM), LBH589 (1.67 nM) and SAHA (2.5 µM), administered for 3 consecutive days, being the results presented in Figures 25 and 26. The drugs concentrations were chosen according to the FMCA results not exerting an effect higher than 50% in the reduction of cellular viability.

697 cell line demonstrated to be more sensitive than KOPN8 to the combination of drugs, as expected from monotherapy results (Figure 23 and 24). In both cell lines, the percentage of cell viability, for the combination of LBH589 and SAHA with 5-AC, never exceeded the percentage of cell viability from 5-AC in monotherapy, indicating that these combinations are not very effective. Otherwise, the combinations of Panobinostat and Vorinostat with Decitabine demonstrated to decrease cell viability more than monotherapy, on both cell lines. 697 cells maintained the highest sensibility to treatment, with lower % of viable cells, when compared with KOPN8 cells. KOPN8 cells, after 72h of incubation, showed lowest % of viable cells for the three schemes of combinations studied, however, none demonstrated to decrease significantly cell viability when compared with monotherapy.

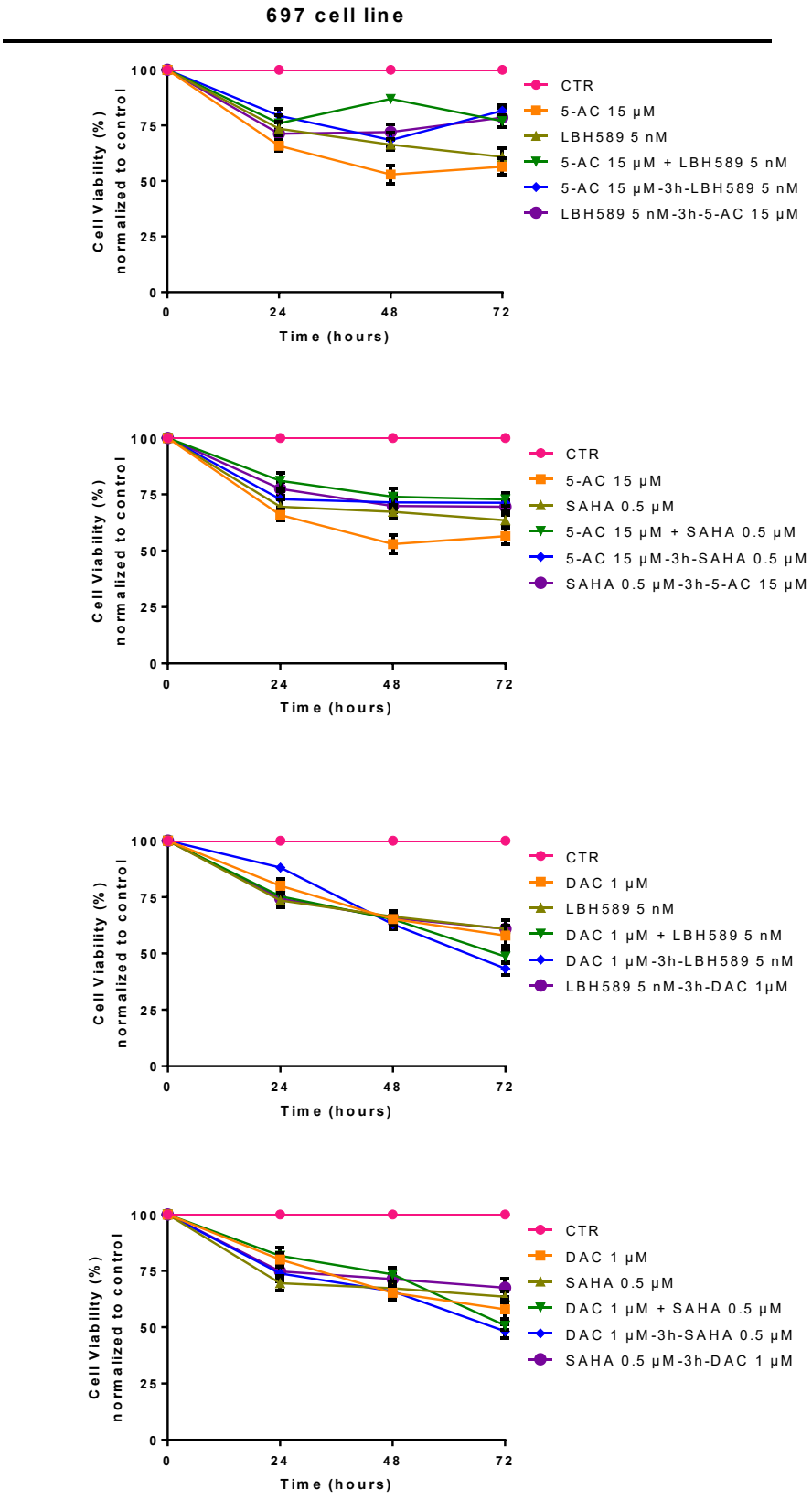

Figure 23 - Dose response curves of the Azacytidine, Decitabine, Panobinostat and Vorinostat in combination therapy in 697 cell line. 697 cell line was incubated for 72h, in the absence (control) and presence of 5-AC, DAC, LBH589 and SAHA in monotherapy and combination in three different therapeutic schemes, as indicated in figure. Dose response curves were established by FMCA method each 24h, as described in Methods. Results are expressed in percentage (%) normalized to control. Data are expressed as mean ± SEM obtained from at least 8 independent experiments. CTR correspond to cells with no treatment. Control, CTR; Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA

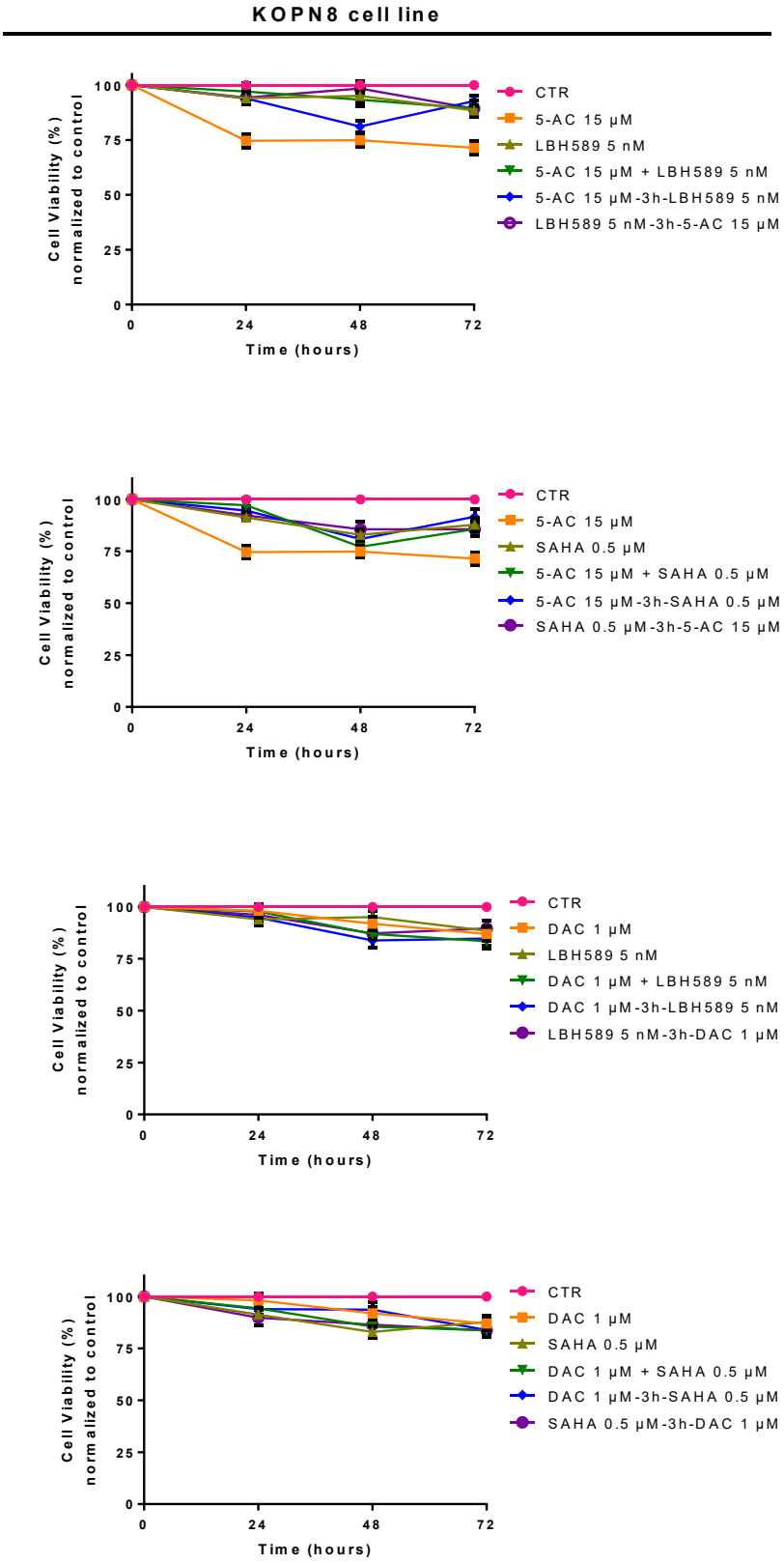

Figure 24 - Dose response curves of the two hypomethylating agents and the two histone deacetylase inhibitors in combination therapy in KOPN8 cell line. KOPN8 cell line was incubated for 72h, in the absence (Control) and presence of 5-AC, DAC, LBH589 and SAHA in monotherapy and combination in three different therapeutic schemes, as indicated in figure. Dose response curves were established by FMCA method each 24h, as described in Methods. Results are expressed in percentage (%) normalized to control. Data are expressed as mean ± SEM obtained from at least 8 independent experiments. CTR correspond to cells with no treatment. Control, CTR; Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA

The therapeutic scheme demonstrated to have an influence on cell viability, with lowest % of viable cells reached with the administration of LBH589 3 hours after DAC as well SAHA 3 hours after DAC, when compared with the other therapeutic schemes tested. However, the decrease was not considered significant. Therefore, these two combinations were used for the flow cytometry studies.

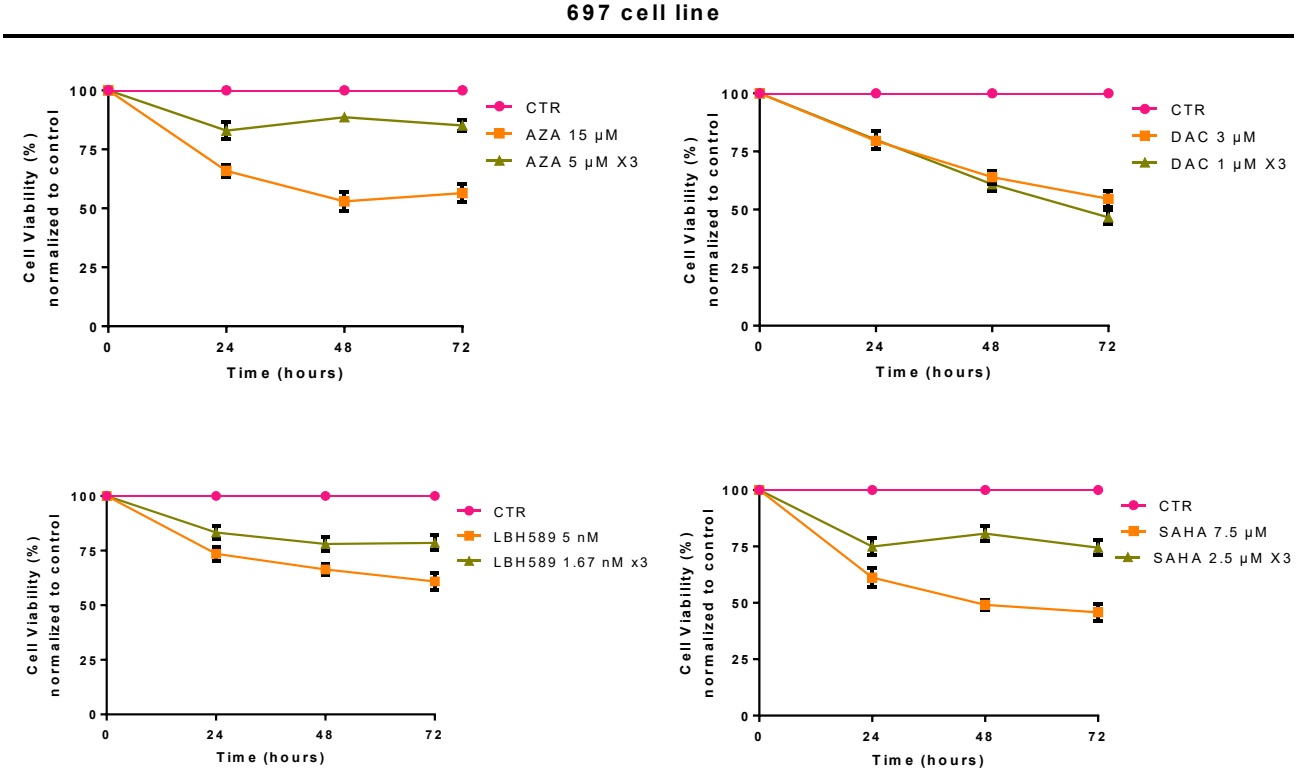

Figure 25- Dose response curves of epidrugs in a daily dose administration in 697 cell line. 697 cell line was incubated for 72h, in the absence (control) and presence of 5-AC, DAC, LBH589 and SAHA in monotherapy and daily dose administration of 3 days, as indicated in figure. Dose response curves were established by FMCA method each 24h, as described in Methods. Results are expressed in percentage (%) normalized to control. Data are expressed as mean ± SEM obtained from at least 8 independent experiments. CTR correspond to cells with no treatment. Control, CTR; Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA

Daily dose administration data (Figure 25 and 26) demonstrated that only de administration of three fractionated doses of DAC on 697 cells showed better results, with a decrease of viable cells of approximately 10% when compared with monotherapy, however it was not significant.

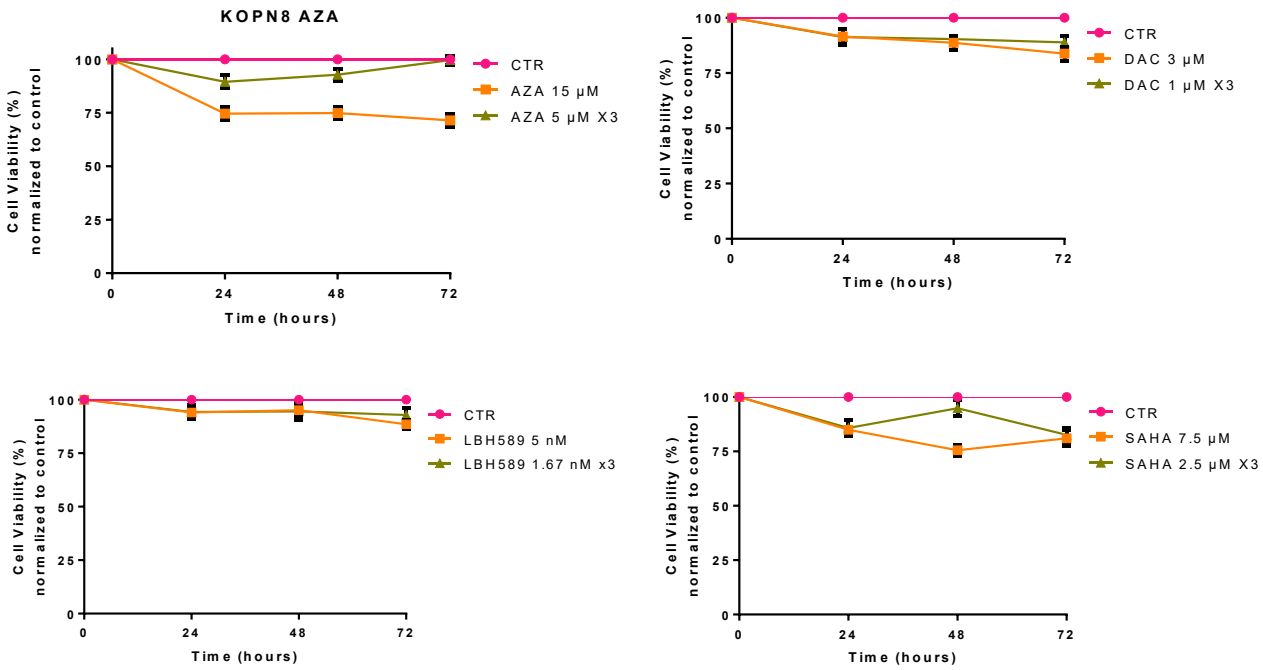

Figure 26 - Dose response curves of epidrugs in a daily dose administration in KOPN8 cell line. KOPN8 cell line was incubated for 24h, 48h and 72h, in the absence or in the presence of 5-AC, DAC, LBH589 and SAHA in monotherapy and daily dose administration of 3 days, as indicated in figure. Dose response curves were established by FMCA method each 24h, 48h, and 72h, as described in Methods. Results are expressed in percentage (%) normalized to control. Data are expressed as mean ± SEM obtained from at least 8 independent experiments. CTR correspond to cells with no treatment. Control, CTR; Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA

3.1.4. The effect on cell death of DNA hypomethylating agents (Azacytidine and Decitabine) and histone deacetylase inhibitors (Panobinostat and Vorinostat) in 697 and KOPN8 cell lines

Anticancer drugs demonstrated to have a cytotoxic effect that promotes cell death. Therefore, it is important to study the exact type of death induced by these epidrugs in use. The detection of cell death type, induced in cells after administration of 5-AC, DAC, LBH589 and SAHA, were obtained by flow cytometry, as described in Methods. After 72h of incubation, cells were labelled with annexin V and Propidium Iodide and the results are represented in Figure 27. As mentioned in section 3.1.3, only the two combinations with best results in dose-response curves were used in flow cytometry studies (administration of LBH589/SAHA and DAC, with an interval of 3h).

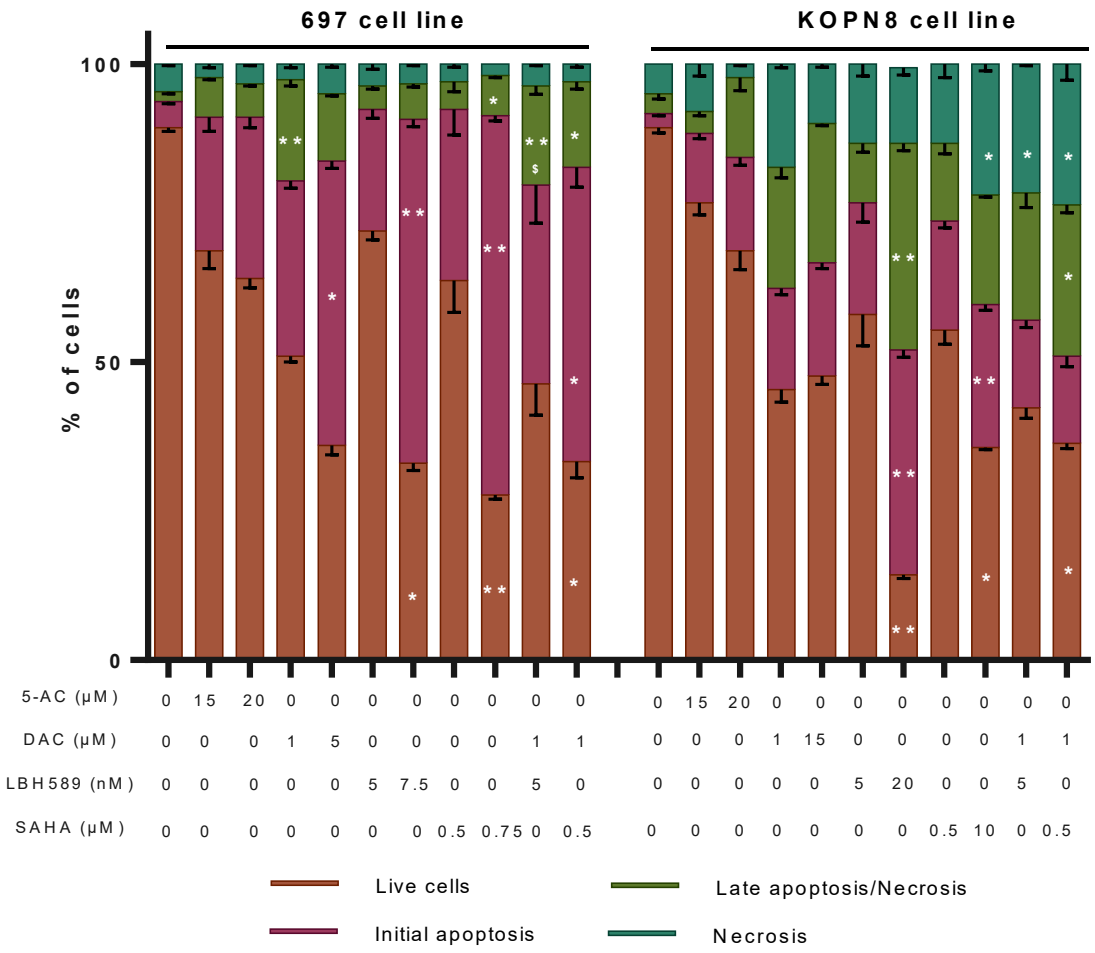

Figure 27 – Evaluation of cell death in 697 and KOPN8 cells by flow cytometry. 697 and KOPN8 cells lines were incubated for 72 hours with 5-AC, DAC, LBH589 and SAHA at the concentrations reported in figure. Cell death were studied by flow cytometry as reported in Methods. The results represent the mean ± standard error of mean (SEM) of 3 independent experiments, which are expressed as percentage (%) of cells at each group. The combinations tested in flow cytometry are the last two columns and represent DAC-3h-LBH589 and DAC-3h-SAHA, respectively, in both cell lines. * $p<0.05$ comparing with control; ** $p<0.01$ comparing with control; $ $p<0.05$ comparing with LBH589 in monotherapy. Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA

In terms of % of cells, all studied drugs (5-AC, DAC, LBH589, SAHA) promoted a decrease in 697 cell viability inducing cell death mostly by apoptosis. However, in KOPN8 cells, DAC, LBH589 and SAHA, induced death by apoptosis and necrosis. Data obtained for 697 cells, after 72h of incubation, demonstrated that only the IC_{50} concentrations of DAC (5 µM), LBH589 (7.5 nM), SAHA (0.75 µM) and the combination of DAC and SAHA induced significant cell death by apoptosis when compared with control, $p<0.05$, $p<0.01$, $p<0.01$ and $p<0.05$, respectively. Cell death by late apoptosis/necrosis was significantly induced on 697 cells when treated with DAC (1 µM), Vorinostat (0.75 µM) and the two combinations tested. As for KOPN8 cells, after 72h of incubation, the IC_{50} concentration of LBH589 (20 nM) showed statistically significance for the induction of apoptosis ($p<0.01$), compared with control, as well as for SAHA (1 µM), with $p<0.01$. The two combinations and SAHA (1 µM) demonstrated to induce significant necrosis ($p<0.05$), when compared with control cells.

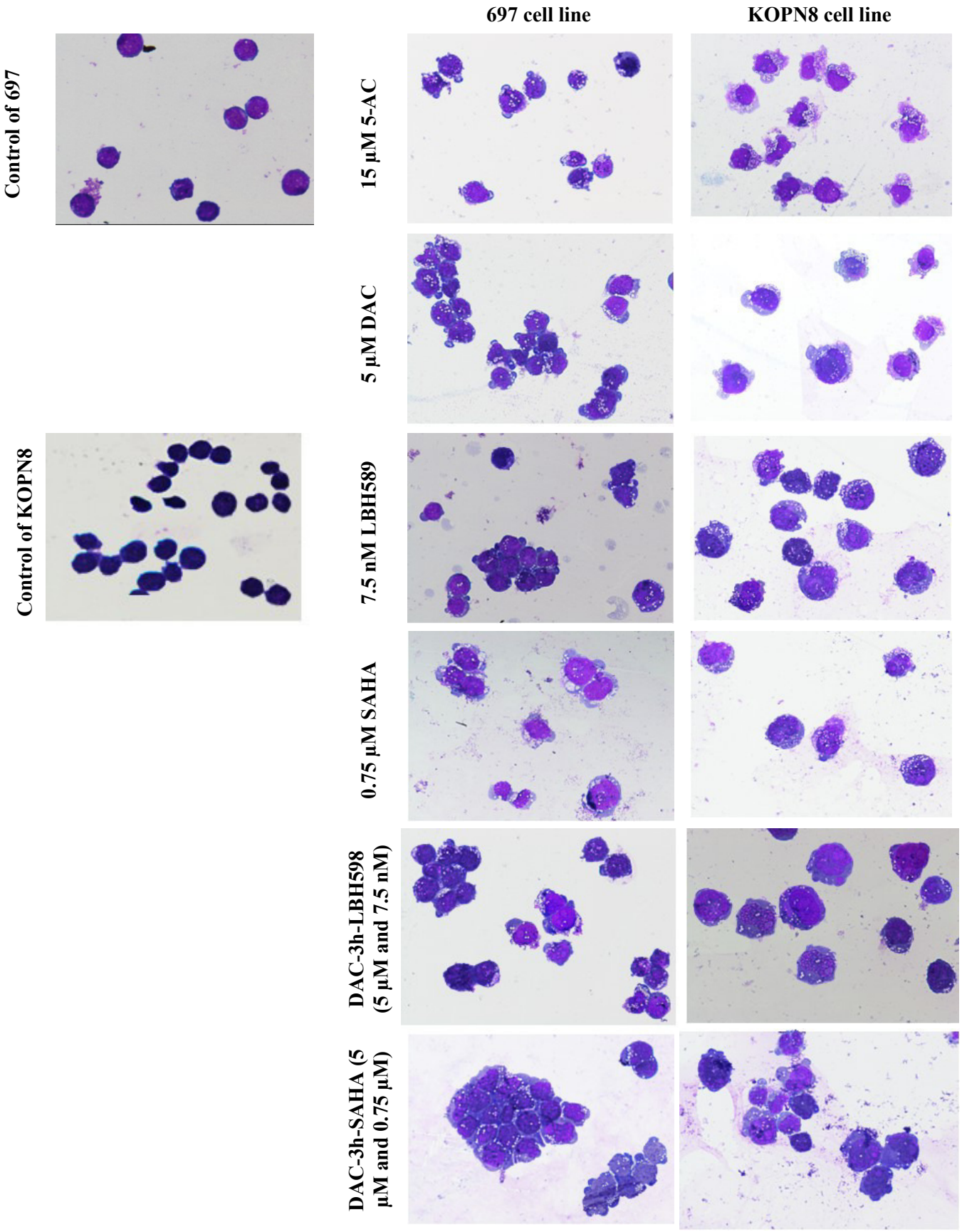

Figure 28 - Morphologic aspect of 697 and KOPN8 cell lines in the absence (control) and presence of the epidrugs. The morphologic aspect of the cells was evaluated after May-Grunewald-Giemsa coloration, as explained in Methods, and the microscope slides were observed by optical microscopy. All images were amplified 500x. It is possible to observe plasma membrane projections (blebbing) that are characteristic of the apoptotic process, indicating that apoptosis is the type of cell death that all four epidrugs induce. Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA

The results of cell death by apoptosis were confirmed by optical microscopy in all the studied conditions. However, it was not possible to detect death by necrosis on the smears of KOPN8 cells treated with SAHA (1 μM) and the two combinations. As observed in Figure 28, cells treated with drugs in monotherapy and in combination showed the presence of morphological characteristics of apoptosis as nuclear contraction and plasma membrane projections (blebbing). We also observed cell vacuolization, which might be related with apoptosis or autophagy.

3.1.5. The effect on cell cycle of DNA hypomethylating agents (Azacytidine and Decitabine) and histone deacetylase inhibitors (Panobinostat and Vorinostat) in 697 and KOPN8 cell lines

In addition to the cytotoxic effect, anticancer drugs may also have an anti-proliferative (cytostatic) effect. The effect on cell cycle of 697 and KOPN8 cells, after administration of 5-AC, DAC, LBH589 and SAHA, were obtained by flow cytometry, as described in Methods. After 72h of incubation, cells were labelled with PI and the results are represented on Table 4. As mentioned in section 3.1.3, only the two combinations with best results in dose-response curves were studied in flow cytometry (administration of LBH589/SAHA and DAC, with an interval of 3h).

All tested conditions, referred in Table 4, showed an increase in the percentage of cells in Sub G_0/G_1 column compared with control values, indicating the presence of apoptosis, which corroborate the results obtained previously by flow cytometry and optical microscopy.

Furthermore, almost all drugs in the several tested conditions seemed to cause cell cycle arrest of KOPN8 cells in G_0/G_1 phase. The exception was the 20 μM concentration of Azacytidine, that induced cell arrest in G_2/M phase. As for 697 cells, the two concentrations tested of 5-AC and DAC have a tendency to induce cell arrest between S and G_2/M phase; LBH589 and SAHA induced significant cell arrest in G_2/M phase ($p<0.05$). The two combinations tested induced cell arrest in different phases of cycle in 697 cells, with the combination of Panobinostat and Decitabine to demonstrate having a tendency to induce cell arrest in G_0/G_1 phase and the combination of Vorinostat and Decitabine inducing significant cell arrest in G_2/M phase ($p<0.05$).

Table 4 - Cell cycle study in 697 and KOPN8 cells.

	Condition	Sub G_0/G_1	G_0/G_1	S	G_2/M
697	CTR	0.0 ± 0.0	50.3 ± 2.0	40.3 ± 1.5	9.3 ± 0.7
	5-AC 15 µM	2.0 ± 1.5	43.3 ± 2.6	47.7 ± 4.4	9.0 ± 2.0
	5-AC 20 µM	6.0 ± 1.0	38.3 ± 0.3	46.7 ± 1.2	15.0 ± 1.2
	DAC 1 µM	9.3 ± 3.2	42.3 ± 2.2	44.0 ± 2.1	13.7 ± 1.3
	DAC 5 µM	5.3 ± 0.3	43.3 ± 0.3	41.0 ± 0.0	15.7 ± 0.3
	LBH589 5 nM	1.3 ± 0.7	51.3 ± 3.3	33.7 ± 2.7	15.0 ± 1.0
	LBH589 7.5 nM	6.0 ± 0.6	32.7 ± 0.3 *	43.7 ± 0.3	23.7 ± 0.3 *
	SAHA 0.5 µM	2.0 ± 0.6	41.3 ± 1.8	37.7 ± 2.7	21.0 ± 1.5 *
	SAHA 0.75 µM	4.3 ± 0.3	32.7 ± 0.3 *	56.3 ± 0.3	11.0 ± 0.6
	DAC-3h-LBH589 (1 µM / 5 nM)	15.7 ± 1.5 ** $	55.7 ± 2.9	34.7 ± 3.2	9.7 ± 0.7
	DAC-3h-SAHA (1 µM / 0.5 µM)	13.0 ± 0.6 **	36.3 ± 3.7	42.0 ± 7.6	21.7 ± 4.1*
KOPN8	CTR	1.3 ± 0.7	36.7 ± 2.7	55.3 ± 2.4	8.0 ± 2.0
	5-AC 15 µM	1.7 ± 0.3	44.3 ± 1.5	45.0 ± 0.6	10. 7 ± 1.2
	5-AC 20 µM	2.7 ± 0.9	34.0 ± 1.2	49.3 ± 0.7	16.7 ± 1.8
	DAC 1 µM	13.0 ± 2.6	41.0 ± 0.6	49.3 ± 1.7	9.3 ± 1.5
	DAC 15 µM	18.3 ± 1.5	37.7 ± 0.3	56.3 ± 0.3	6.0 ± 0.6
	LBH589 5 nM	9.3 ± 4.9	40.0 ± 2.1	50.3 ± 3.7	9.7 ± 3.8
	LBH589 20 nM	36.7 ± 1.2 *	37.7 ± 0.9	59.3 ± 0.7	3.0 ± 0.6
	SAHA 0.5 µM	12.0 ± 4.4	49.7 ± 1.9	42.7 ± 0.3	7.7 ± 1.7
	SAHA 1 µM	37.7 ± 1.2 **	54.3 ± 0.7 *	33.7 ± 0.7*	12.0 ± 1.2
	DAC-3h-LBH589 (1 µM / 5 nM)	17.7 ± 3.7	44.3 ± 1.5	45.3 ± 3.3	10.7 ± 2.4
	DAC-3h-SAHA (1 µM / 0.5 µM)	22.0 ± 3.5	51.3 ± 1.2	39.7 ± 1.2	9.0 ± 0.0

697 and KOPN8 cell lines were incubated for 72 hours with 5-AC, DAC, LBH589 and SAHA at the concentrations reported in the table. Cell cycle phases were studied by flow cytometry. The results represent the mean ± standard error of mean (SEM) of 3 independent assays, which are expressed as percentage (%) of cells at each stage of the cell cycle.
* $p<0.05$ comparing with control; ** $p<0.01$ comparing with control; *** $p<0.001$ comparing with control;
$ $p<0.05$ comparing with LBH589 in monotherapy.
Control, CTR; Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA.

3.2. Methylation studies on 697 and KOPN8 cell lines (before and after treatment)

As mentioned before, 5-mC detection reflects the methylation levels of cells. Therefore, 5-mC levels were determined and analysed by flow cytometry as indicated in Methods. Results are expressed in Figure 29.

Figure 29 shows that KOPN8 cells have slightly higher levels of 5-mC compared with 697 cells and treatment with epigenetic modulators induce a decrease of those levels, and consequently the methylation process, more accentuated in KOPN8 cells. However, the levels of 5-mC of cells treated with the two histone deacetylase agents were very similar to control.

The decrease of 5-mC levels were statistically significant only for KOPN8 cells treated with the two DNA hypomethylating agents (Azacytidine and Decitabine), with a decrease of MIF of approximately 76.8% ($p<0.05$) compared with control (44.3% MIF/191% MIF) and 79.6% ($p<0.01$) compared with control (39% MIF/191% MIF), respectively. As for 697 cells, only the ones treated with 20 µM of Azacytidine ($p<0.05$) showed a significant decrease of MIF, approximately 57.5% compared with control (153.6% MIF/168% MIF).

Although the combinations tested in this category presented lower MIF values than monotherapy, they were not statistically significant. However, the two DNA hypomethylating agents decreased 5-mC levels, whereas the two histone deacetylase agents were very similar to control.

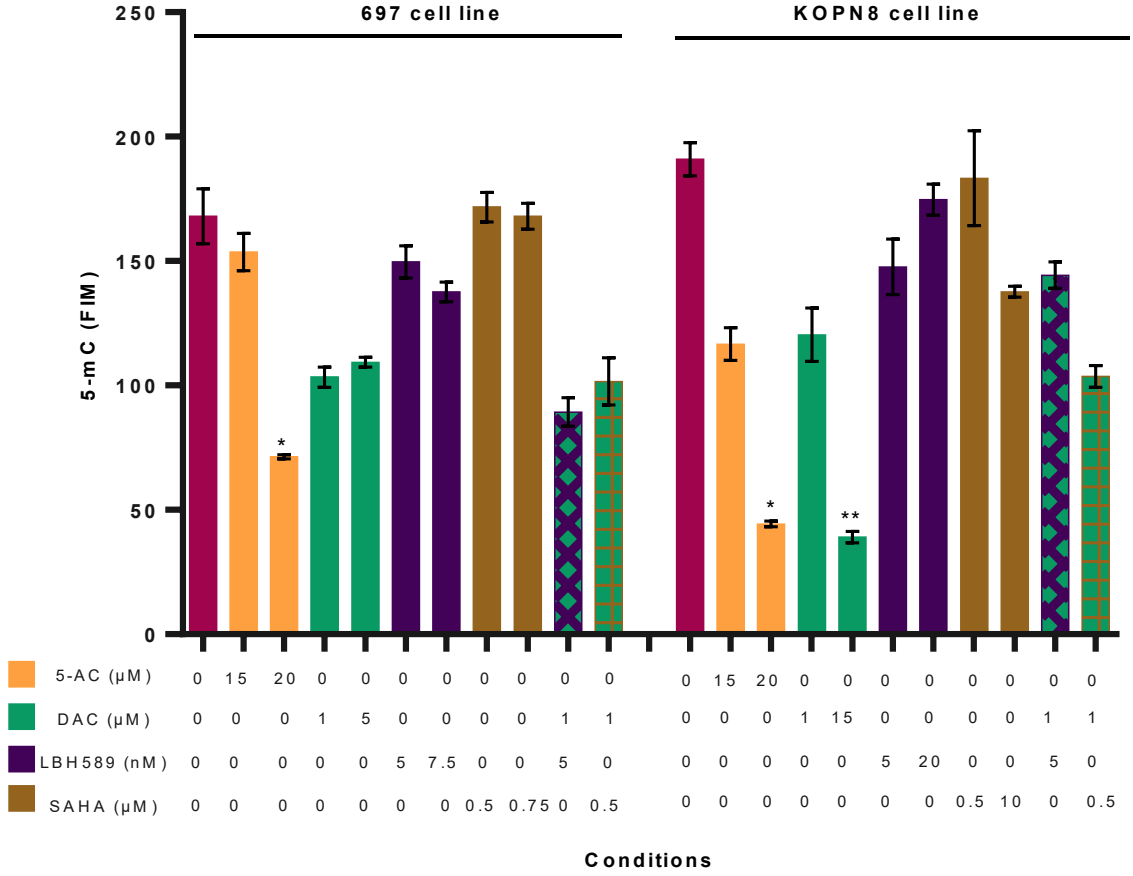

Figure 29 – Evaluation of 5-mC levels in 697 and KOPN8 cells treated with hypomethylating agents and histone deacetylase inhibitors. 697 and KOPN8 cell lines were incubated for 72 hours with 5-AC, DAC, LBH589 and SAHA at the concentrations reported in figure. 5-mC detection was studied by flow cytometry as reported in Methods. The results represent the mean ± standard error of mean (SEM) of 3 independent assays, expressed as MFI (Mean Fluorescence Intensity Mean) at each condition. The combinations tested are the last two columns and represent DAC-3h-LBH589 and DAC-3h-SAHA, respectively, in both cell lines. * $p<0.05$ comparing with control; ** $p<0.01$ comparing with control. Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA; Administration of DAC and LBH589 with 3h interval, DAC-3h-LBH589; Administration of DAC and SAHA with 3h interval, DAC-3h-SAHA.

Since it was observed a decrease in 5-mC levels, the DNA methylation status of 25 tumour suppressor genes (*TP73, MSH6, VHL, RARB, ESR1, CDKN2A, PAX5, KLLN, MGMT, PAX6, WT1, CD44, GSTP1, ATM, CADM1, CHFR, BRCA2, RB1, THBS1, PYCARD, CDH13, TP53, BRCA1, STK11* and *GATA5*) was analysed by MS-MLPA, in order to identify genes that suffered alteration on their methylation status after treatment (Tables 5 and 6). The results were obtained from three replicates for each cell line and each condition.

Table 5 – Gene methylation profile of KOPN8 cells by MS-MLPA.

KOPN8 cell line

Gene ID	CTR line	5-AC (15µM)	DAC (1µM)	LBH589 (5nM)	SAHA (0.5µM)	DAC-3h-LBH589 (1µM+5nM)	DAC-3h-SAHA (1µM+0.5µM)
TP73	0	0	0	0	0	0	0
MSH6	33.5 ± 20.5	26.5 ± 2.1	26.5 + 16.3	32 ± 25.5	45 ± 14.1	45.5 ± 20.5	35 ± 14.1
VHL	0	0	0	0	0	0	0
RARB	67.5 ± 2.1	67 ± 2.8	70.5 ± 2.1	69 ± 0	66 ± 7.1	69 ± 2.8	66.5 ± 0.7
ESR1	97.5 ± 0.7	96.5 ± 2.1	90.5 ± 3.5	99.5 ± 2.1	92 ± 2.8	90 ± 1.4	95.5 ± 3.5
CDKN2A	0	0	0	0	0	0	0
PAX5	10.5 ± 7.8	7 ± 0	10 ± 0	16 ± 0	14 ± 7.1	16 ± 9.9	10.5 ± 6.4
KLLN	16.5 ± 13.4	12.5 0.7	11.5 ± 7.8	17 ± 7.1	23.5 ±12.1	25.5 ± 17.7	17.5 ± 9.2
MGMT	23 ± 4.2	17 ± 4.2	19.5 ± 4.9	24.5 ± 12.1	25.5 ± 3.5	24.5 ± 4.9	21.5 ± 2.1
PAX6	95.5 ± 0.7	103.5 ± 3.5	94 ± 11.3	97.5 ± 3.5	94 ± 0	94.5 ± 0.7	94 ± 1.4
WT1	63 ± 2.8	59.5 ± 2.1	61 ± 5.7	64.5 ± 0.7	64 ± 1.4	64 ± 0	62.5 ± 2.1
CD44	21.5 ± 0.7	28 ± 8.4	22 ± 1.4	21 ± 1.4	21.5 ± 0.7	21 ± 0	21 ± 0
GSTP1	0	0	0	0	0	0	0
ATM	0	0	0	0	0	0	0
CADM1	96 ± 2.8	102 ± 1.4	99.5 ± 3.5	95 ± 2.8	99.5 ± 0.7	94 ± 2.8	93 ± 0
CHFR	0	0	0	0	0	0	0
BRCA2	0	0	0	0	0	0	0
RB1	0	0	0	0	0	0	0
THBS1	89.5 ± 2.8	87 ± 4.2	90.5 ± 3.5	92.5 ± 0.7	88.5 ± 3.5	92 ±2.8	89 ± 2.8
PYCARD	0	0	0	0	0	0	0
CDH13	85 ± 2.1	96 ± 8.4	82.5 ± 12.1	86 ± 2.8	83 ± 0	83 ± 0	83 ± 1.4
TP53	0	0	0	0	0	0	0
BRCA1	0	0	0	0	0	0	0
STK11	38.5 ± 4.9	26.5 ± 4.9	37 ± 2.8	37.5 ± 4.9	41 ± 4.2	35.5 ± 2.1	38 ± 1.4
GATA5	64.5 ± 2.1	72 ± 5.7	67.5 ± 0.7	64 ± 5.7	66 ± 2.8	64 ± 5.7	65.5 ± 3.5

KOPN8 was incubated for 72 hours with monotherapy and combination of drugs, at the concentrations reported in the table. Then, DNA was extracted by salting out protocol, as explained in methods. Methylation patterns were studied by MS-MLPA. The results represent the mean ± standard error of mean (SEM) of 3 independent assays, which are expressed as percentage (%) of methylation at each condition. CTR line – control for the line in study; Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA; administration of LBH589 3 hours after DAC, DAC-3h-LBH589; administration of SAHA 3 hours after DAC, DAC-3h-SAHA.

Table 6 – Gene methylation profile of 697 cells by MS-MLPA.

	697 cell line						
Gene ID	**CTR line**	**5-AC (15µM)**	**DAC (1µM)**	**LBH589 (5nM)**	**SAHA (0.5µM)**	**DAC-3h-LBH589 (1µM+5nM)**	**DAC-3h-SAHA (1µM+0.5µM)**
TP73	102 ± 2.8	89 ± 15.5	74 ± 22.6	94 ± 5.7	101 ± 4.2	77.5 ± 19.1	81 ± 18.4
MSH6	31.5 ± 21.9	34.5 ± 6.4	29.5 ± 13.4	41 ± 15.6	27 ± 19.8	35.5 ± 6.4	26.5 ± 4.9
VHL	0	0	0	0	0	0	0
RARB	95.5 ± 0.7	94 ± 1.4	89 ± 5.7	94 ± 5.7	95 ± 1.4	92 ± 12.7	90.5 ± 7.8
ESR1	98 ± 1.4	99.5 ± 3.5	98 ± 1.4	93 ± 5.7	97 ± 2.8	87 ± 1.4	92.5 ± 9.2
CDKN2A	91.5 ± 4.9	81.5 ± 10.6	66 ± 22.6	94 ± 1.4	92 ± 8.5	77 ± 24.0	77.5 ± 21.9
PAX5	13 ± 0	12.5 ± 0.7	7.5 ± 3.5	14 ± 8.5	11	9 ± 4.2	7.5 ± 0.7
KLLN	16 ± 9.9	20 ± 5.7	15 ± 7.1	24.5 ± 13.4	14.5 ± 10.6	18.5 ± 3.5	13 ± 0
MGMT	100 ± 2.8	80.5 ± 27.6	72.5 ± 30.4	94.5 ± 3.5	97.5 ± 9.2	73.5 ± 23.3	76 ± 22.6
PAX6	69 ± 2.8	71 ± 8.5	66.5 ± 3.5	59.5 ± 12.1	68.5 ± 3.5	69 ± 4.2	66.5 ± 10.6
WT1	57 ± 2.8	56 ± 1.4	57.5 ± 4.9	50 ± 8.5	56.5 ± 2.1	56.5 ± 2.1	56.5 ± 3.5
CD44	95 ± 1.4	81 ± 8.5	79.5 ± 20.5	96.5 ± 3.5	96 ± 0	85 ± 26.9	80.5 ± 14.8
GSTP1	0	0	0	0	0	0	0
ATM	0	0	0	0	0	0	0
CADM1	80.5 ± 3.5	87.5 ± 2.1	78 ± 1.4	81 ± 4.2	77 ± 0	81 ± 4.2	74 ± 8.5
CHFR	0	8 + 0	0	0	0	0	0
BRCA2	0	0	0	0	0	0	0
RB1	0	0	0	0	0	0	0
THBS1	94 ± 2.8	95.5 ± 0.7	89 ± 4.2	94 ± 7.1	94 ± 4.2	93.5 ± 0.7	91.5 ± 0.7
PYCARD	16 ± 2.8	16 ± 0	12.5 ± 4.9	17 ± 0	16.5 ± 3.5	14.5 ± 2.1	12.5 ± 2.1
CDH13	110 ± 2.8	105.5 ± 3.5	99.5 ± 4.9	102.5 ± 4.9	104 ± 7.1	90 ± 5.7	94.5 ± 10.6
TP53	0	0	0	6 ± 0	0	0	0
BRCA1	0	0	0	0	0	0	0
STK11	91 ± 1.4	83.5 ± 0.7	83 ± 14.1	94 ± 0	95 ± 11.3	85.5 ± 10.6	79 ± 7.1
GATA5	96 ± 2.8	95.5 ± 2.1	89.5 ± 10.6	97.5 ± 2.1	99.5 ± 2.1	95 ± 7.1	90.5 ± 4.9

697 was incubated for 72 hours with monotherapy and combination of drugs, at the concentrations reported in the table. Then, DNA was extracted by salting out protocol, as explained in methods. Methylation patterns were studied by MS-MLPA. The results represent the mean ± standard error of mean (SEM) of 3 independent assays, which are expressed as percentage (%) of methylation at each condition. CTR line – control for the line in study; Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA; administration of LBH589 3 hours after DAC, DAC-3h-LBH589; administration of SAHA 3 hours after DAC, DAC-3h-SAHA.

None of the conditions in study demonstrated to have a demethylating effect on 697 and KOPN8 cells. The two DNA hypomethylating agents and the two histone deacetylase inhibitors demonstrated to have a similar effect on gene methylation, since none of the conditions tested seem to decrease significantly the methylation percentage of the 25 tumour suppressor genes tested. 14 out of the 17 genes hypermethylated in 697 cells (*MSH6, RARB,*

ESR1, PAX5, KLLN, MGMT, *PAX6*, *WT1, CD44, CADM1, THBS1, CDH13, STK11* and *GATA5*) are also hypermethylated in KOPN8 cells. However, *TP73*, *CDKN2A* and *PYCAR*D are only hypermethylated in 697 cells.

As represented in Table 5, in cells treated with 5-AC we observed a decrease in the methylation levels of *MSH6* and *WT1,* while in cells treated with DAC a decrease in the methylation levels of *MSH6* is also detected as well as in *ESR1,* but with no statistical significance. The histone deacetylase inhibitors and the combination of the two kinds of epigenetic modulators did not change the frequency of methylation of genes in KOPN8 cells, except for *ESR1* and *CADM1* promoter genes. However, the results are not statistically significant compared with control data. As observed in Table 6, in 697 cells, all conditions tested decreased the methylation levels of *TP73, MGMT* and *CDH13*. Azacytidine, Decitabine and the two combinations tested decreased the methylation levels of *CDKN2A, CD4* and *STK11*. DAC alone and the association with SAHA decreased the methylation levels of *THBS1* and *GATA5*. *MSH6* and *CADM1* suffered a decrease on its methylation levels in cells treated with DAC, SAHA alone and in combination with DAC. DAC also decreased the methylation levels of *RARB* and *PAX6* and LBH589 decreased the % of methylation of *ESR1*, *PAX6* and *WT1*. The combination of LBH589 and DAC also decreased the methylation levels of *RARB* and *ESR1,* and the combination of SAHA and DAC decreased de methylation levels of *RARB*, *ESR1* and *PAX6*. However, none of the decreases mentioned above demonstrated statistical significance when compared with cell line control.

3.3.Evaluation of the therapeutic potential of epigenetic modulators in CLL patients

As studied in ALL cell lines, the therapeutic effect of the four epigenetic modulators were also studied on CLL patient samples. To access the therapeutic potential of hypomethylating agents and histone deacetylase inhibitors, PBMCs were incubated in the absence (control) and presence of 5-AC and DAC, during 48h and cell viability results are presented in Figure 30.

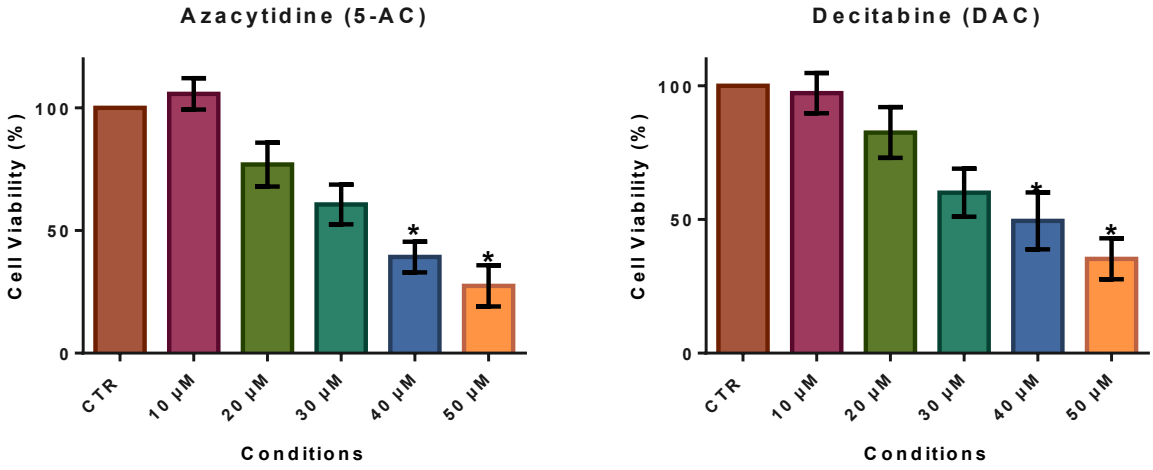

Figure 30 – Dose response curves of 5-AC and DAC administered in monotherapy in CLL patients. CLL mononuclear cells from 11 patients were incubated for 48h, in the absence or in the presence of increasing concentrations of Azacytidine and Decitabine in monotherapy, as indicated in figure. Dose response curves were established by FMCA method, as described in Methods. Results of cell viability are expressed in percentage (%). Data are expressed as mean ± SEM. CTR correspond to cells with no treatment. Control, CTR.

As we can observe in Figure 30, 5-AC and DAC induced a decrease in cell viability on a dose dependent manner, in CLL patients. CLL cells demonstrated to reduce significantly its viability for 50% and lower, when treated with concentrations of 5-AC and DAC higher than 30 µM ($p<0.05$), when compared with control.

PBMCs were also incubated in the absence (control) and presence of LBH589 and SAHA, during 48h, with data presented in Figure 31.

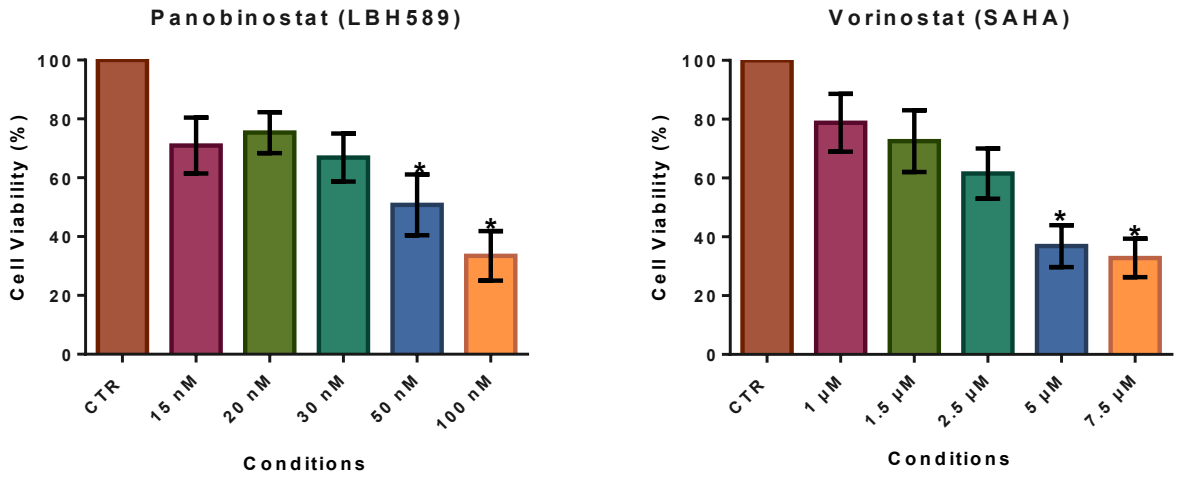

Figure 31 - Dose response curves of LBH589 and SAHA administered in monotherapy in CLL patients. CLL mononuclear cells from 11 patients were incubated for 48h, in the absence or in the presence of increasing concentrations of Panobinostat and Vorinostat in monotherapy, as indicated in figure. Dose response curves were established by FMCA method, as described in Methods. Results of cell viability are expressed in percentage (%). Data are expressed as mean ± SEM. CTR correspond to cells with no treatment. Control, CTR.

CLL cells reduced its cell viability when treated with increased doses of LBH589 and SAHA and both drugs demonstrated to have an effect on cell viability dependent of drug concentration. Doses higher than 30 nM for LBH589 demonstrated to reduce significantly cell viability in about 60% ($p<0.05$). Vorinostat induced a significant decrease (>50%) in CLL cell viability only with concentrations higher than 2.5 µM ($p<0.05$).

The combination of drugs and the administration of each drug in a daily dose scheme permit the achievement of the same therapeutic effect with lowest doses, compared to those used in monotherapy, providing a better response with lower toxicity and consequently, less side effects so characteristic of high dosages. Thus, the effect of combination and daily dose administration was determined by FMCA technique, as explained in Methods, with the purpose of evaluating their effect on cell viability. Cells were incubated in the absence (control) and presence of 5-AC (30 µM), DAC (30 µM), LBH589 (15 nM) and SAHA (1 µM), for 48h, being the effect of combination presented in Figure 32 and those for daily dose administration in Figure 33. In this therapeutic scheme, cells were incubated with 5-AC (15 µM), DAC (15 µM), LBH589 (25 nM) and SAHA (1.25 µM) for 48h, being administered for 2 consecutive days.

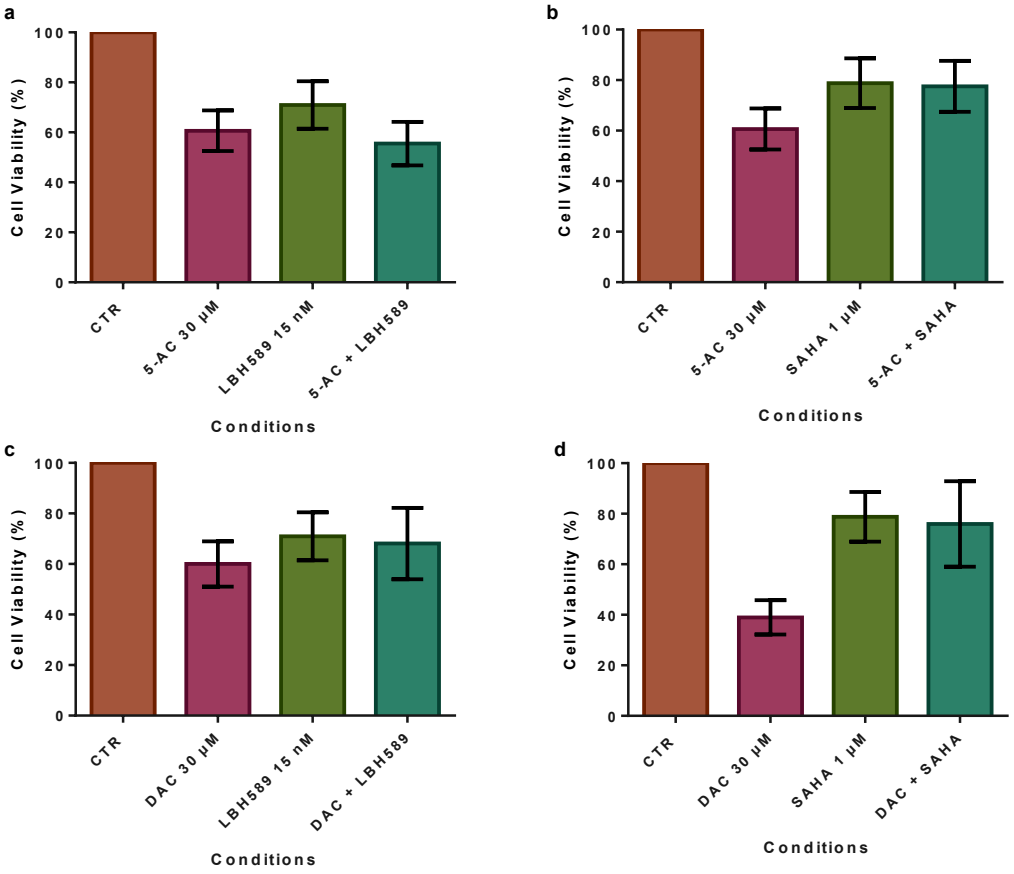

Figure 32 - Dose response curves of the epidrugs in combination therapy in CLL patients. CLL mononuclear cells form 11 patients were incubated for 48h, in the absence or in the presence of 5-AC, DAC, LBH589 and SAHA in monotherapy and combination. Dose response curves were established by FMCA method as described in Methods. Results are expressed in percentage (%) of cell viability. Data are expressed as mean ± SEM. Control, CTR; Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA

The study of drug combination demonstrated that independently of the combination tested none demonstrated to be statistically significant, in comparison to monotherapy. 5-AC in combination with LBH589 obtained better results on cell viability than the ones obtained for monotherapy doses, with a decrease on the % of viable cells, however, it was not significant.

Similarly to the combination therapeutic schemes, the daily dose administration (Figure 33) of 5-AC, DAC, LBH589 and SAHA did not induce a higher reduction on cell viability comparatively to the same concentrations in single dose administration.

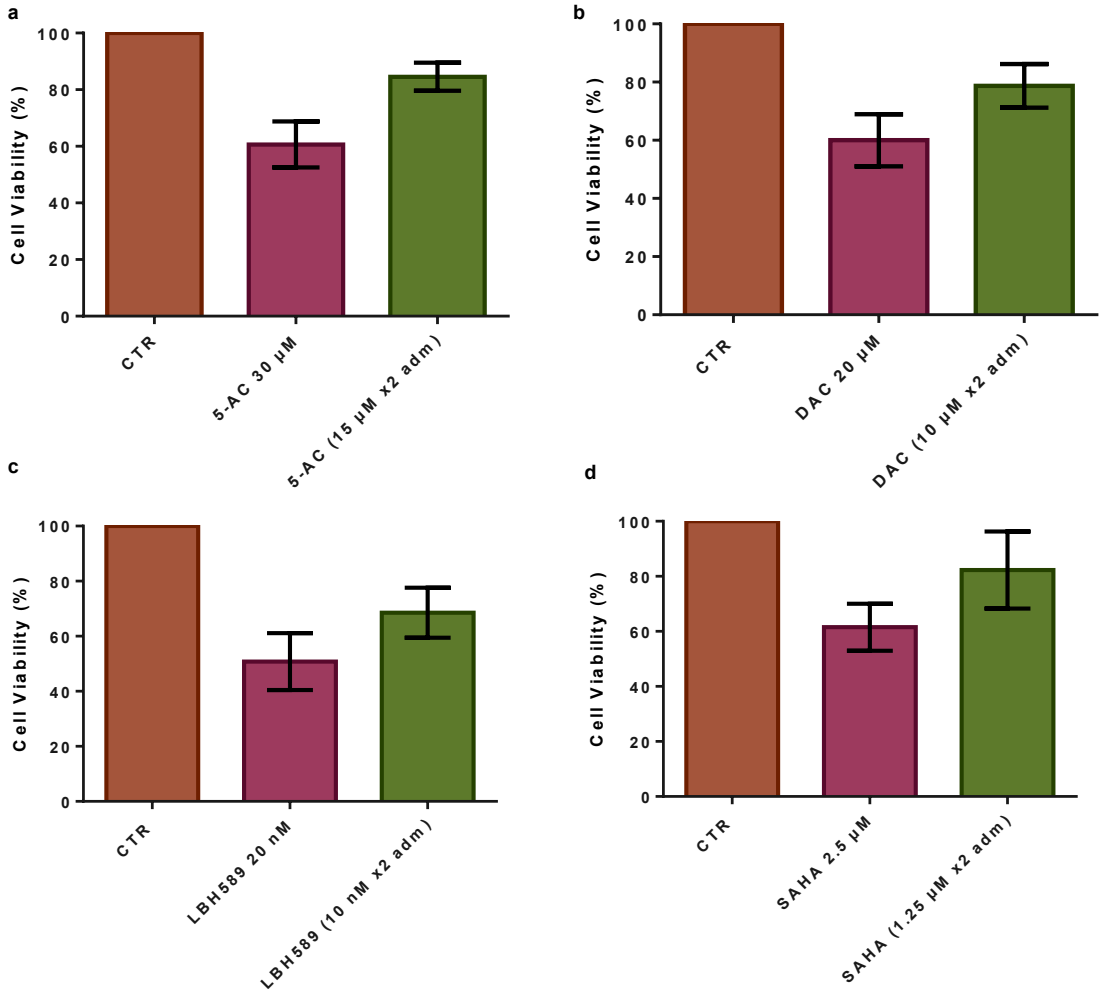

Figure 33 - Dose response curves of the epidrugs in a daily dose administration in CLL patients. CLL mononuclear cells form 11 patients were incubated for 48h, in the absence or in the presence of 5-AC, DAC, LBH589 and SAHA in monotherapy and daily dose administration of 2 days, as indicated in figure. Dose response curves were established by FMCA, as described in Methods. Results are expressed in percentage (%) of cell viability. Data are expressed as mean ± SEM. CTR correspond to cells with no treatment. Control, CTR; Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA

3.3.1. The effect of DNA Hypomethylating agents (Azacytidine and Decitabine) and histone deacetylase agents (Panobinostat and Vorinostat) on CLL cell death

The study of the therapeutic effect of the four epigenetic modulators enrolled in this project were already presented and demonstrated to have effect on CLL patient cells, however it is important to understand if this drugs target specifically neoplastic cells. For that, the detection of the cell type that are affected by the epigenetic therapies in study were obtained by flow cytometry and the results are presented in Figure 34. After 48h of incubation with 5-AC, DAC, LBH589 and SAHA with the IC_{50} concentrations, cells were labelled with annexin V and CD5 and CD19 antibodies, as described in Methods, in order to distinguish the effect on neoplastic lymphocytes versus non-neoplastic cells.

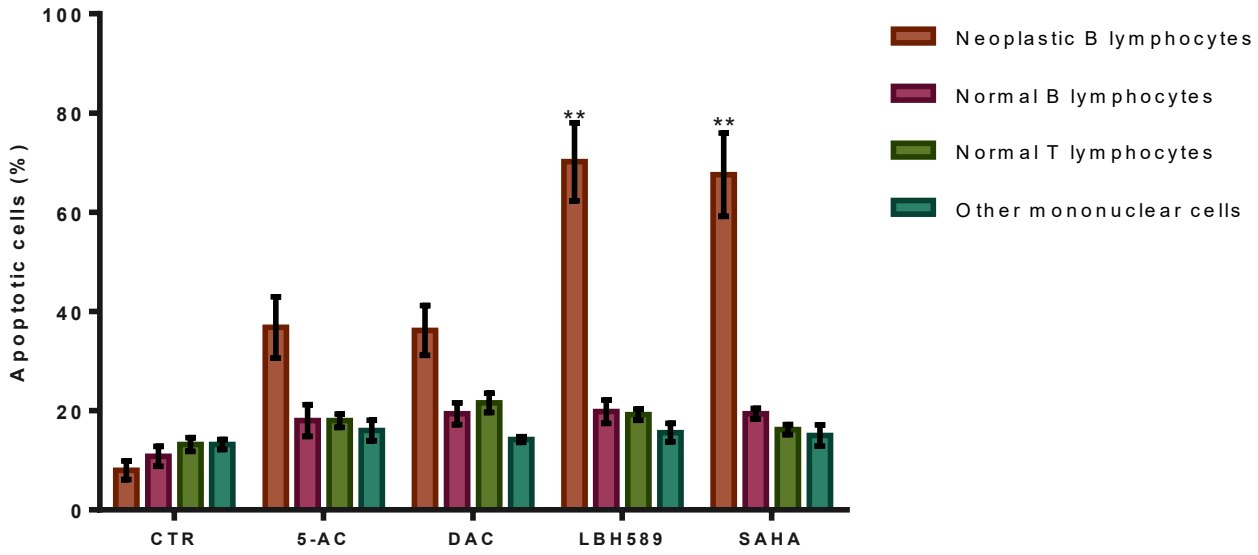

Figure 34 - Evaluation of cell death, induced by 5-AC, DAC, LHB589 and SAHA in CLL patients. Mononuclear cells from 11 patients were incubated for 48 hours with the IC_{50} dose of 5-AC (20µM-60 µM), DAC (15 µM -60 µM), LBH589 (15nM-125nM) and SAHA (1 µM -10 µM). IC_{50} doses were calculated for every patient individually. Cell death were studied by flow cytometry as reported in Methods. The results represent the mean ± standard error of mean (SEM,) which are expressed as percentage (%) of death cells at each group. ** p<0,01 comparing with control; Control, CTR; Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA

As we can observe in the figure, all four drugs demonstrated to induce an increase in apoptosis of neoplastic B lymphocytes when compared with normal lymphocytes and the other types of mononuclear cells in tested samples. However, the results are statistically significant only when cells are treated with LBH589 and SAHA at IC_{50}, compared with control ($p<0.01$), with an increase of apoptotic cells in about 60%. The two hypomethylating agents also induced apoptosis of about 35% of cells, 15% higher than control, however it was not significant.

3.3.2. The effect on cell cycle of DNA hypomethylating agents (Azacytidine and Decitabine) and histone deacetylase inhibitors (Panobinostat and Vorinostat)

The anticancer drugs not only have a cytotoxic effect on mononuclear cells, but also has an anti-proliferative (cytostatic) one. The study of the effect on cell cycle, after administration of 5-AC, DAC, LBH589 and SAHA, were obtained by flow cytometry, as described in Methods. After 48h of incubation, cells were labelled with PI/RNase and the results are presented in Table 7.

The induction of apoptosis, observed on cell death studies, was confirmed by the presence of a Sub G_0/G_1 peak, but mostly on cell treated with the two histone deacetylase inhibitors, LBH589 and SAHA, which have higher and statistically significant results, with 30.4% ($p<0.01$) and 24.4% ($p<0.05$) of cells, respectively. All the epigenetic modulators demonstrated a tendency to interrupt cell cycle in S phase. However, only in cells treated with LBH589 and SAHA, the differences were statistically significant ($p<0.01$), with increased % of cells in that phase, 25.6% and 23.8%, respectively, when compared with control (5.4%).

Table 7 – Cell cycle study on CLL mononuclear cells by flow cytometry.

	Condition	Sub G_0/G_1	G_0/G_1	S	G_2/M
CLL	CTR	3.6 ± 0.8	91.0 ± 1.3	5.4 ± 0.9	3.6 ± 0.7
	5-AC 20-60 µM	5.2 ± 0.9	83.0 ± 2.9	11.8 ± 2.7	5.2 ± 0.5
	DAC 15-60 µM	8,.6 ± 2,5	80.0 ± 3.9	14.8 ± 3.6	5.2 ± 0.7
	LBH589 15-125 nM	30.4 ± 3.4 **	70.2 ± 4.0 **	25.6 ± 4.3 **	4.2 ± 0.7
	SAHA 1-10 µM	24.40 ± 3.5 *	70.2 ± 2.9 **	23.8 ± 3.1 **	6.0 ± 1.0

Mononuclear cells were incubated for 48 hours with 5-AC, DAC, LBH589 and SAHA at the concentrations range reported in the table. Cell cycle phases were studied by flow cytometry. The results represent the mean ± standard error of mean (SEM), which are expressed as percentage (%) of cells at each stage of the cell cycle.
* $p<0.05$ comparing with control; ** $p<0.01$ comparing with control. Control, CTR; Azacytidine, 5-AC; Decitabine, DAC; Panobinostat, LBH589; Vorinostat, SAHA

3.4.Study of the methylation patterns of CLL samples

The present study enrolled 21 CLL patients with a median age of 70 years (range 50 – 87), 24% (n = 5) females and 76% (n = 16) males. Regarding Rai staging system, 10 (47.6%) were low risk (Rai 0), 5 (23.8%) were intermediate risk (Rai I and II) and 6 (28.6%) were high risk (Rai III and IV). The control group consisted of 10 subjects without haematological malignancies with a median age of 66.4 years (range 57 – 80), 30% females (n = 3) and 70% males (n = 7). There were no statistical differences in biodemographic characteristics between cases and control. The DNA methylation status of the 25 tumour suppressor genes (*TP73, MSH6, VHL, RARB, ESR1, CDKN2A, PAX5, KLLN, MGMT, PAX6, WT1, CD44, GSTP1, ATM, CADM1, CHFR, BRCA2, RB1, THBS1, PYCARD, CDH13, TP53, BRCA1, STK11* and *GATA5*) was analysed by MS-MLPA, according to manufacturer's instructions. Results are presented in Figures 35 and 36.

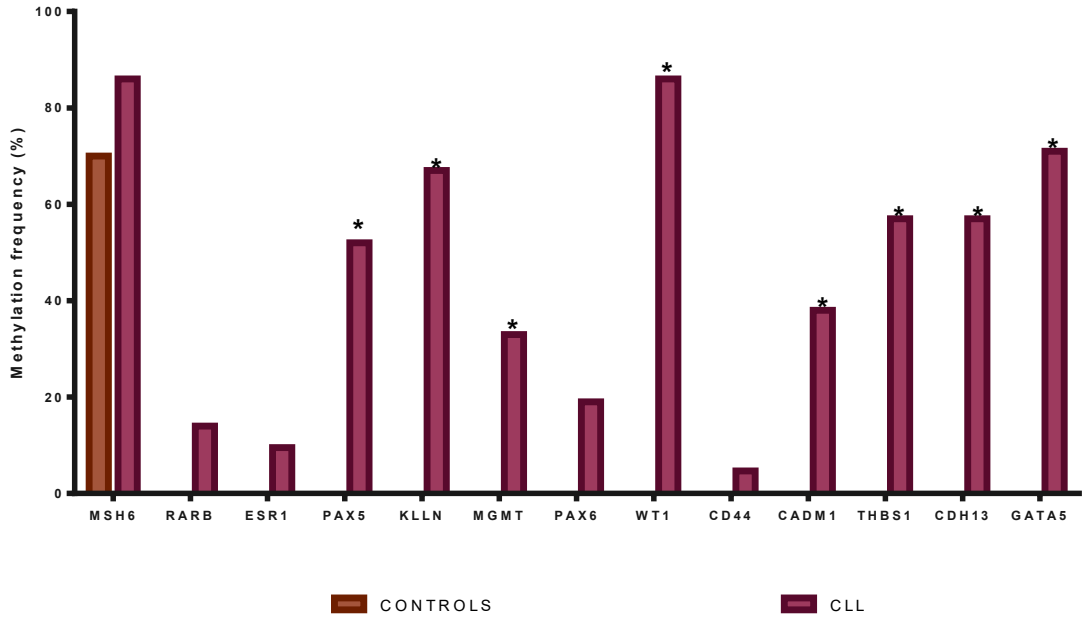

Figure 35 – Methylation profile in controls and CLL patients. Methylation frequency of 25 tumour suppressor genes were analyzed by MS-MLPA in 21 patients, as described in Methods, and were compared between controls and CLL patients. Only methylated genes are represented. Results are presented as methylation frequency (%). * $p<0.05$ comparing with control.

As observed in Figure 35, CLL patients had a significant higher methylation frequency of *MSH6* (86%, 18/21), *WT1* (86%, 18/21), *GATA5* (71%, 15/21) and *KLLN* (67%, 14/21) gene promoters, when compared with controls, that only had *MSH6* (70%, 7/10) gene promoter significantly methylated. CLL patients demonstrated to have some other methylated genes with a lower methylation frequency such as *THBS1* (57%, 12/21), *CDH13* (57%, 12/21), *PAX5* (52%, 11/21), *CADM1* (38%, 8/21) and *MGMT* (33%, 7/21). Furthermore, all CLL patients had at least one methylated gene.

Next, we analysed the methylation status according to RAI staging system (Figure 36). This analysis showed that high risk CLL patients have higher methylation frequency of *MSH6* gene promoter [low risk: 36.4% (4/11); intermediate risk: 66.7% (2/3); high risk: 100% (7/7); p<0.01]. *THBS1* gene promoter also demonstrated to be very methylated in high risk group compared with low risk [low risk: 45.5% (5/11); intermediate risk: 33.3% (1/3); high risk: 85.7% (6/7), however, the difference was not considered statistically significant. *MSH6* gene promoter did not show clinical significance when comparing patients and controls, since it was hypermethylated on both groups. However, it shows clinical significance when comparing risk groups, being highly methylated on CLL high risk group (Rai III and IV).

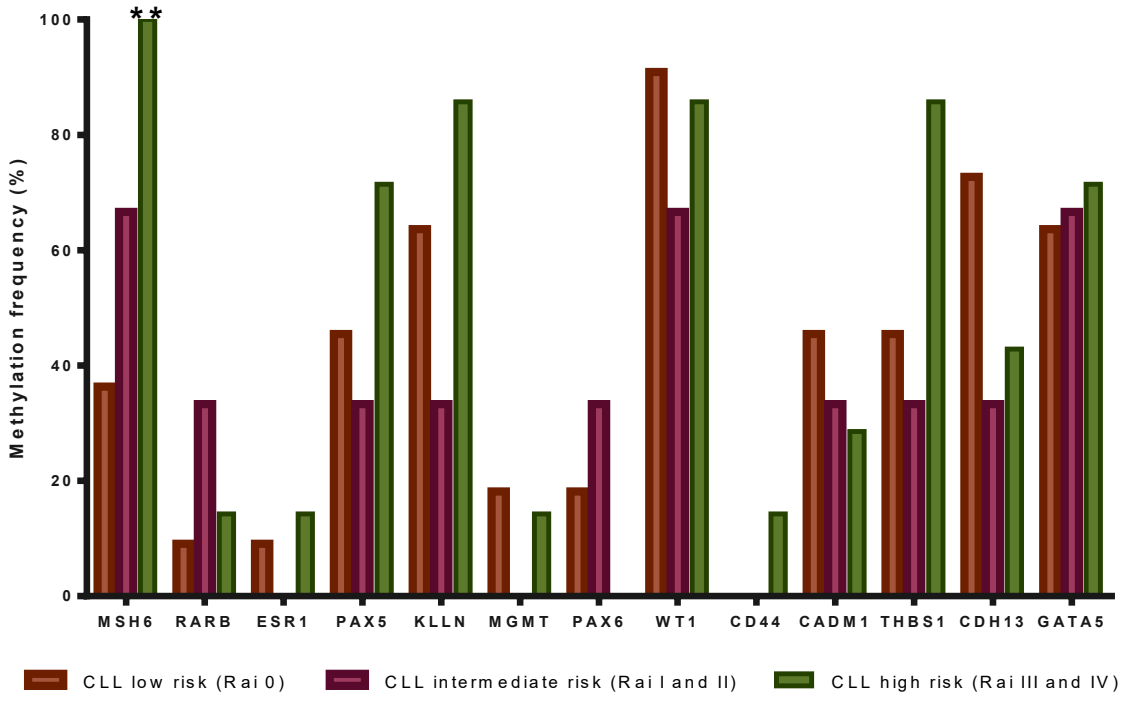

Figure 36- Methylation profile in CLL patients according to Rai staging system. Methylation frequency of 25 tumour suppressor genes were analyzed by MS-MLPA in 21 patients, as described in Methods, and were compared between CLL risk groups. Only methylated genes are represented. Results are presented as methylation frequency (%). ** p<0.01 comparing with low risk group.

4 Discussion

ALL and CLL are two types of leukaemia of the lymphoid lineage that can affect both B and T lymphocytes. While in ALL cells predominates a deregulation in cell differentiation and proliferation, CLL cells demonstrated to be resistant to apoptosis (8,20). Despite ALL being a disease of the younger and CLL affecting older people, both implicate epigenetic alterations that somehow may be involved in their development. DNA methylation and histone deacetylation are two types of epigenetic modifications that are important for the normal functioning of the organism since they regulate gene expression and may induce DNA condensation, respectively. The deregulation of those processes promotes malignant alterations in cells. DNA methyltransferases and HDAC enzymes are responsible for gene silencing of important genes in ALL and CLL diseases (45,56,135,140). Therefore, the study of the effect of DNA hypomethylating agents and histone deacetylase inhibitors in ALL and CLL diseases seem to be a good start to identify new therapeutic approaches for these malignancies.

Prior to this study, there are limited investigation using Azacytidine, Decitabine, Panobinostat and Vorinostat in patients with ALL and CLL and clinical trials using the combination of drugs are also limited, according to clinical trial databases. Reviewing those databases, it is observed that Azacytidine is being studied in combination with chemotherapy in ALL patients, some studies completed and other still ongoing (149,172); Decitabine is being studied alone but also in combination with chemotherapy, and in combination with Vorinostat, and also all combined, Decitabine, Vorinostat and chemotherapy (173–175). Panobinostat and Vorinostat are also being studied for their possible use in ALL treatment, alone or in combination with other already in use drugs, inclusive chemotherapy (146,176). In CLL patients the scenario is very similar; Azacytidine is being studied to treat CLL, alone or in combination with other drugs such as Lenalidomide, Venetoclax, Rituximab and Romidepsin (177,178,148); Decitabine is being tested alone or in combination with valproic acid, Vorinostat and Romidepsin for the treatment of CLL (179,147,180); Panobinostat is also being studied for CLL treatment (181); Vorinostat is being studied alone or in combination with other drugs such as Cyclophosphamide and Rituximab in CLL patients (182). Despite all, combinations of DNA methyltransferase inhibitors with histone deacetylase inhibitors have not been reposted in clinical studies, being necessary many contributes to this area.

All four drugs studied in this project, two DNA hypomethylating agents (Azacytidine and Decitabine) and two histone deacetylase inhibitors (Panobinostat and Vorinostat) are in use for clinical practice in other types of cancers and it is important to understand their cellular effect on other type of cells as it was studied in this project. Azacytidine was approved by FDA and EMA to treat MDS and CMML; Decitabine is used to treat MDS; Panobinostat is administered to treat multiple myeloma; Vorinostat is used for the treatment of cutaneous T-cell lymphoma. Their therapeutic potential has been studied in diseases mentioned above, and according to literature their effect is slightly different, dependent to the type of disease, not being possible to correlate with the results obtained in this study. However, it is important to confirm some general aspects of their action.

Initially in this project, it was studied the therapeutic potential of the two DNA hypomethylating agents (5-AC and DAC) and the two histone deacetylase inhibitors (LBH589 and SAHA). All four tested drugs demonstrated to

have effect on cell viability in the two B-ALL cell lines, dependent to concentration (increasing doses decrease cell viability), incubation time (increased time of incubation decrease cell viability), and cell type (697 cell line seem to be more sensitive to all four drugs, since the IC_{50} concentration was reached with lower doses compared with KOPN8 cell line, where IC_{50} dose was not reached with tested doses). Other important fact that it was observed in some concentrations at some time of incubation was the reverse effect on cell viability. Some concentrations after some time of incubation demonstrated to lose its effect on cells, being observed an increase in their proliferation. This may indicate that it is necessary to reinforce the administration of some dugs to increase its effect on cell viability. One example is the possible need of the reinforcement of 5-AC administration, by administrating another dose after 24h and 48h to increase 5-AC effect on cell viability. DAC demonstrated to have more effect than 5-AC, when comparing same doses, in 697 cell line, being possible to conclude that despite of its similar mechanism of action they can have slightly different effects, possibly due to different genetic backgrounds of cell lines. 5-AC and DAC are structurally related but at the same time distinct, 5-AC is a ribonucleoside and can be incorporated both into DNA and RNA, and DAC is a deoxyribonucleoside, being incorporated solely into DNA, as described before (Figure 10) (151,153). Once incorporated into DNA, 5-AC and DAC have similar mechanisms of action, including depletion of DNMTs, hypomethylation of DNA, and induction of DNA damage. However, the mechanisms of action that might explain differences in clinical activities of 5-AC and DAC have not been clearly defined. The fact that DAC show better results may be due to its fastest activation compared with Azacytidine, since Decitabine only suffers two phosphorylations and 5-AC need to be phosphorylated, then reduced and phosphorylated again to be incorporated into DNA, as explained above (Figure 10). Another explanation may be due to the "dual mechanism" of DAC, as Hollenbach *et al.* (2010) explained in their research on human AML cell lines (THP-1, HL-60, KG-1 and OCI-AML3) (183). Hollenbach *et al.* (2010) compared the effect of 5-AC and DAC (0.02–50 μM for both drugs) on cell viability after 72h and observed that DAC had more effect on cells when compared with same doses of 5-AC (DAC affected cells at concentrations 2- to 10-fold lower than 5-AC), due to greater incorporation into DNA (183). This fact was also observed in this project, since DAC also demonstrated to have more effect on 697 cells, when comparing same doses of 5-AC. Hollenbach *et al.* (2010) also observed that Azacytidine incorporation into RNA and DNA showed a distribution of 65% and 35%, respectively. This fact may explain why DAC is more potent than 5-AC since it is only incorporated into DNA. An explanation of the different results for both similar drugs, given by researchers, may be due to the "dual mechanism" of DAC that have been referred to inhibit cell proliferation at high doses and cause DNA hypomethylation and consequently gene re-expression at low doses (184). This dual mechanism affects important processes of cell differentiation, tumour suppression and also activates some immune mechanisms.

Next, it was studied the effect of daily dose administration to understand if the administration of fractionated doses of concentrations tested in monotherapy were beneficial. The results demonstrated that none of the drugs tested induce more effect on cell viability than the one observed in monotherapy, not being beneficial this type of administration scheme, at least at studied concentrations.

After understanding that daily dose administration was not useful for the treatment of 697 and KOPN8 cells, it was studied the combination of drugs. In theory, combining drugs or administering them fractionally may be beneficial because it can decrease the side effects, since it is administered lower doses (185). However, in this case the results show that the two ALL cell lines tested do not benefit mutual action. 697 cell line also demonstrated to be more sensitive than KOPN8 cells, as observed in monotherapy studies. KOPN8 cell line did not benefit from combinations of drugs, obtained similar values of cell viability, of the ones observed for monotherapy, at least at tested doses. On the other hand, 697 cells obtained lower levels of cell viability than monotherapy when treated with the combination of LBH589 with DAC and SAHA with DAC, independently of the administration scheme. This demonstrate that it is beneficial to combine those drugs, even though the difference was little. The scheme with lowest percentage of viable cells was the administration of LBH589 and SAHA 3 hours after the administration of DAC. The combinations with Azacytidine did not show to be beneficial compared with monotherapy with an effect lower than 5-AC alone. This may be due to errors in the incubation, or the doses testes need to be increased, or in DNA hypomethylating agents and histone deacetylase inhibitors cases, the time needed for them to interact with the cell need to be increased. However, it is unlikely, since cells responded well to monotherapy which in some way validate the data obtained for combination and daily dose administration.

Therefore, combinations and daily administrations schemes do not fit well for DNA hypomethylating agents and histone deacetylase inhibitors. The next step will be to increase doses and time of incubation to confirm if the results are similar.

After realizing that the two DNA hypomethylating agents and the two histone deacetylase inhibitors have a dose and time dependent effect on cell viability, reducing the % of viable cells, it was important to understand if they also had a cytostatic effect. To corroborate the previous results, were performed cell death, cell cycle and morphology studies. Results show that all four drugs cause cell death by apoptosis, in 697 and KOPN8 cell lines. The apoptotic process was also confirmed by changes in morphologic aspects, with the presence of blebbing. Hollenbach *et al.* (2010) also confirmed that both hypomethylating agents induce apoptosis, but DAC continue to produce more effect on cells (183). These results are in agreement with those obtained in on our study. In fact, DAC also demonstrated to have more effect than 5-AC. These results are also confirmed by flow cytometry, as 5-AC and DAC increased the % of cells that are annexin V positive and also the fraction of cells in sub-G_0/G_1 peak. 697 cells showed higher levels of apoptosis, which corroborates the results obtained from dose-response studies where it was observed that 697 was more sensitive to therapy. Panobinostat, a highly potent HDACi, displays antitumor activity against a range of malignancies, particularly haematological diseases, such as MM, cutaneous T-cell lymphoma, Hodgkin lymphoma, and CML, being approved by FDA and EMA to treat MM (186). In a study made in Multiple Myeloma cell lines (RPMI 8226, OPM2, U266, and H929) incubated with three doses of LBH589 (2 nM; 4 nM and 6 nM), it was showed that this drug induces apoptosis. In another studies, Panobinostat also induced apoptosis via mitochondrial dysfunction (187,188). Then, the results obtained for LBH589 on both studies mentioned above corroborate the results obtained in 697 and KOPN8. Vorinostat, another highly potent HDACi, also demonstrated to induce death by apoptosis in the literature, for example, in an *in vitro* study, Silva *et*

al. (2013) studied the effect of Vorinostat in K562, HL60, THP-1 and $CD33^+$ cells from AML and MDS patients and they observed that Vorinostat inhibited growth and induced apoptosis, corroborating the results obtained in our project (189).

The combinations studied by flow cytometry, LBH589 and SAHA administered with DAC, with an interval of 3h, induced significant apoptosis only in 697 cells, when compared with control. In a global way, the two histone deacetylase inhibitors seemed to induce higher levels of apoptosis than the two DNA hypomethylating agents in 697 cell line only. This goes against the expected since DNA hypomethylating agents influence directly the expression of genes, expected to cause more cell death than the histone deacetylase inhibitors, which influence indirectly gene expression by regulating the condensation state of DNA. KOPN8 cells showed similar results of apoptotic levels, independent to treatment. In fact, the different techniques used to, direct or indirect, assess the cell death type were in agreement and reveal statistical significances. All the results emphasize the relevance of inducing apoptosis to treat lymphoproliferative disorders, to reverse the elevated proliferation of malignant cells.

After that, it was studied if these drugs also show effect on cell cycle, causing cell arrest. Besides the cytotoxic effect, hypomethylating agents and histone deacetylase inhibitors also showed an antiproliferative action (cytostatic). For KOPN8 cell line, all conditions, monotherapy and the two combinations, seemed to have a tendency to cause cell cycle arrest in G_0/G_1 phase, except of 5-AC 20 μM that induced cell arrest in G_2/M phase. For 697 cell line, 5-AC induced cell arrest on S and G_2/M phase, DAC, LBH589 and SAHA all induced cell arrest in G_2/M phase. According to literature, the results obtained on cell cycle studies are divergent and dependent to cell type. In a study made in Multiple Myeloma cell lines (RPMI 8226, OPM2, U266, and H929) incubated with three doses of LBH589 (2 nM; 4 nM and 6 nM) did not show to cause cell cycle arrest. However, in another studies, Panobinostat caused cell-cycle arrest at G_1/S phase (187,188). SAHA also demonstrate to display antitumor activity in literature. In a study made in AML-patient derived blasts cultured *ex vivo* and in two cell lines (NB4 and U937 treated with 1.5 μM and 2 μM), Vorinostat caused cell cycle arrest in the G_2/M phase and arrested cells have accumulated DNA double strand breaks, demonstrating that the induction of apoptosis in AML cells were followed by DNA damage and cell cycle arrest (190). These results suggest, again, that the effect on cell cycle is cell dependent, since they are different of the ones observed in our study. The combinations on 697 cells had a tendency to induce cell arrest in G_0/G_1 phase. Data is sustained by the increase of cells in some phase compared with control. According to results obtained for the two cell lines, cell cycle arrest seemed to be dependent to cell type, since the same therapy caused cell cycle arrest in different phases of the cycle.

In the first part of this project we studied the therapeutic potential of the epigenetic modulators. Then we studied the methylation process in a global and specific way, by detection of 5-mC levels with flow cytometry and by MS-MLPA studies, respectively. The methylation studies based on the detection of 5-mC by flow cytometry showed that only 5-AC and DAC alter the methylation levels, as expected, since LBH589 and SAHA act only on histone deacetylation levels, presenting levels of 5-mC very similar than control. The two combinations tested demonstrated lower levels of 5-mC than the ones observed for monotherapy, indicating that histone deacetylase

inhibitors do not act directly on methylation levels but indirectly, by altering chromosome condensation. This alteration allows the incorporation of DNA hypomethylating agents into DNA causing demethylation of gene promoters and consequently expression of genes (191). The demethylation process is the product of the transformation of 5-mC into 5-hmC that can then be converted into cytosine, not being detected by flow cytometry. Methylation studies by MS-MLPA in 697 and KOPN8 cell lines showed that 14 out of the 25 genes tested (*MSH6, RARB, ESR1, PAX5, KLLN, MGMT*, *PAX6*, *WT1, CD44, CADM1, THBS1, CDH13, STK11* and *GATA5*) demonstrated to be methylated in both cell line controls. This high frequency of DNA methylation and the fact that it might be involved in the pathogenesis of the disease was also observed in a study made in 95 patients with childhood ALL, where they showed that 69% of the patients had at least one gene methylated and 40% showed methylation of multiple genes (192). However, 697 cells demonstrated to have three more genes methylated (*TP73, CDKN2A* and *MGMT*) compared with KOPN8 cells, demonstrating somehow the heterogeneity of ALL methylation patterns. The percentage of methylation of the same gene also demonstrated to be different in both cell lines, as observed, per example, for *RARB* gene promoter that showed a higher methylation level in 697 cell line, approximately 90%, and a methylation level of 70% in KOPN8 cell line. Comparing the different conditions tested and the control of the cell line, results did not show significant differences in percentage of methylation, demonstrating no demethylating effect on 697 and KOPN8 cells. It was also not observed a significative difference in methylation levels comparing the DNA hypomethylating agents with the histone deacetylase inhibitors. However, the doses tested might not be sufficient to cause significant differences in methylation patters, since they are lower than IC_{50} doses. The next step will be to test IC_{50} doses and compare with already obtained results to better study the influence of the drugs in the methylation process. We already know that drugs influence the viability of neoplastic cells, probably by alteration of the methylation status of some tumour suppressor genes, and we also demonstrated that many tumour suppressor genes are highly methylated. What we need to demonstrate next is that the methylation levels of those genes decrease effectively with treatment. Another important fact observed was that *CDKN2A, PAX6, CD44, CADM1* and *STK11* gene promoters showed no methylation (0%) in non-neoplastic group compared with high levels of methylation in cell lines, which might implicate their role in B-ALL pathogenesis, being important to study those genes in ALL patient samples.

The studies conducted on ALL cell lines were also performed on CLL patient samples. First, we analysed the cytotoxic effect of both DNA hypomethylating agents (Azacytidine and Decitabine) and histone deacetylase inhibitors (Panobinostat and Vorinostat). PBMCs from CLL patients responded very similar to both DNA hypomethylating agent drugs and the effect was dose-dependent. It was observed a decrease on cell viability under the same levels for the same concentrations. However, they seem to be more sensitive to 5-AC than DAC, since for the highest tested doses 40/50 μM the viability for 5-AC and DAC suffers a difference of 10%, beneficiating 5-AC with lower percentage of cell viability, and consequently more effect. This higher effect of DAC was also observed for the two ALL cell lines tested and in the literature (183). LBH589 and SAHA also demonstrated to cause decrease on cell viability of PMBCs, being this effect also dose-dependent, as well observed in literature (187,190). No combination demonstrated to be beneficial, compared with monotherapy, even though 5-AC in combination with LBH589 obtained slightly better results on cell viability than the ones obtained for monotherapy doses. Daily

dose administration also demonstrated to not be more effective in comparison to monotherapy. Despite these outcomes, and since monotherapy reviled promising results, it is necessary further studies with increased number of patients and increased time of incubation, with daily monitoring to access the daily therapeutic potential. An interesting fact is that in ALL cell lines combinations and daily dose administration also demonstrated to not be beneficial, which may indicate that these classes of drugs do not act well together. However, they are different diseases affecting different stages of lymphoblast cells and the results can not be compared. All four drugs demonstrated to also have an antiproliferative effect on CLL cells, arresting cell cycle in S phase. However, histone deacetylase inhibitors caused higher cell arrest than the DNA hypomethylating agents, as observed in the results obtained from cell cycle study. 5-AC and DAC obtained higher results in S phase, where they act, compared with control, however it was not statistically significant compared with results obtained for LBH589 and SAHA (the DNA hypomethylating agents increased 2 to 3 times the percentage of cells in S phase compared with an increase of 5 times for histone deacetylase inhibitors, comparing with control). In literature, none of the drugs tested cause cell cycle arrest in S phase, at least in those diseases where they are approved (151,183,187,190). In terms of cell arrest, as said before, the results are very different, according to cell type affected in the disease, however, it is known that all four drugs have influence and cause cell arrest independently of the disease studied, corroborating the results obtained in this project.

Cell cycle study also allowed to detect the type of cell death induced by the drugs in study and it was possible to conclude that the cell death process induced by all four drugs was apoptosis, observed by elevated peaks in sub-G_0/G_1 phase that are indicative of apoptotic bodies. As observed in cell arrest, histone deacetylase inhibitors also had more effect on cell death than DNA hypomethylating agents, since they caused an increase of 6 to 8 times the percentage of apoptotic bodies compared with only an increase of 2 times for the two DNA hypomethylating agents tested. This fact was a surprise since we know that DNA hypomethylating agents are the ones to affect directly the activation of tumour suppressor genes and consequently the apoptotic process, so it was expected that those class of drugs had the best results in cell death studies. Therefore, it is important to study the reason why histone deacetylase inhibitors had better results, by, for example, study best their mechanism of action and the pathways where they may act. For now, with these data we may only presume that CLL patients are more sensitive to histone deacetylase inhibitor drugs. According to literature, all drugs cause apoptosis no matter the studied disease (183,187,190), corroborating our results.

After supposing that the histone deacetylase inhibitors and the DNA hypomethylating agents induce cell death by apoptosis with the elevated sub-G_0/G_1 peaks, it was studied which type of cells these drugs affect, if only neoplastic or if it affects also normal cells. The studies demonstrated that neoplastic B lymphocytes were more affected by apoptosis than normal B and T cells, which indicates that all drugs target mostly, if not only, neoplastic cells, not being very aggressive to normal cells (the increase of apoptosis compared with control was very small on the other types of cells). This is extremely important because when administered anti-cancer therapies the main concern is not to target normal cells but only neoplastic ones, reducing side effects. The next step will be to study

the effect of previous treatment with conventional therapy on the effect of the two DNA hypomethylating agents and the two histone deacetylase inhibitors.

Therefore, we also studied the methylation process in CLL samples, only by MS-MLPA technique, contrarily to ALL cell lines. These studies showed methylation in 13 out of the 25 tumour suppressor genes studied (*MSH6, RARB, ESR1, PAX5, KLLN, MGMT, PAX6, WT1, CD44, CADM1, THBS1, CDH13, GATA5*), and 8 of these genes (*MSH6, PAX5, KLLN, WT1, CADM1, THBS1, CDH13, GATA5*) were hypermethylated, comparing with control group of non-neoplastic individuals. The highest methylation frequency was observed for *MSH6, KLLN, WT1* and *GATA5* tumour suppressor genes suggesting the involvement of DNA methylation on CLL development, as observed also in ALL. Furthermore, all CLL patients had at least one methylated gene, indicating that this process is very frequent in this type of disease. In literature, this process is reported as also being very frequent in CLL patients as observed in this project, with patients having at least one abnormally methylated gene (193). Next, we analysed the methylation status according to RAI staging system which showed that high risk CLL patients have higher methylation frequency of only one gene promoter, *MSH6*. *THBS*1 gene promoter also demonstrated to be very methylated in high risk group compared with low risk, though, the difference was not considered statistically significant. However, these epigenetic abnormalities may not be important in risk stratification since low- and high-risk patients had similar methylation profiles. An interesting fact observed during these studies was the methylation of *MSH6* promoter gene that did not show clinical significance for the patient vs control study, since it is hypermethylated in both groups. However, in the risk stratification study this gene showed to be hypermethylated in the high risk group. This may indicate that *MSH6* can not be used for diagnosis but it can be used for prognostic purposes. Nevertheless, the results obtained in this project need to be validated in a higher cohort and correlated with additional disease characteristics.

5 Future perspectives and conclusion

Nowadays, investigations are being focused on the development of new therapeutic approaches for the treatment of cancer since it is a global problem of public health. Therefore, the present study aimed to contribute to that investigation.

Our study suggests that all four epigenetic modulators have cytotoxic and cytostatic effects on both ALL cell lines and CLL cells, inducing cell death by apoptosis in a time- and/or dose-dependent manner. This effect was observed in monotherapy and in combination therapy. Thus, it was found that 697 cells are more sensitive to treatment with any of the epidrugs tested, whereas KOPN8 cells resisted to treatment. With combination, in some cases, such as the combination of Decitabine with Panobinostat and Vorinostat, we achieved a significant cytotoxic and cytostatic effect with lower doses of each compound. These results support the possibility of increase effects and at the same time minimizes the secondary toxicity associated with combination of multiple drugs, offering

more therapeutic benefits with potentially less side-effects. Thus, these results revealed that DNA hypomethylating agents and histone deacetylase inhibitors may be promising therapeutic strategies in ALL and CLL treatment.

Methylation results suggested that methylation of tumour suppressor genes is a common event in CLL patients and perhaps may be used to diagnose this disease. It was also observed the methylation of one gene that may be used for prognosis (*MSH6*) which inspire further studies in this area. In ALL cell lines this process was also very frequent, however results need to be confirmed in ALL patient samples.

Further studies of combination and daily dose administration are necessary on ALL cell lines, with increased doses to conclude if in some cases the combination of drugs and its daily dose administration are beneficial or if the results with higher doses are similar to the ones already obtained, discarding the possibility that the inefficiency of combinations are due to low tested doses. It is also important to study some molecules involved in the apoptotic process to corroborate the results obtained from flow cytometry. For the methylation study, it is necessary to test the IC_{50} doses to obtain better methylation patters for each drug tested and it is also important to study the possible role of the identified hypermethylated genes in cancer pathogenesis. To confirm the results obtained in cell lines and study the influence of genetics and individual heterogeneity it is important to reproduce the study made in ALL cells in ALL patient samples.

For CLL it is important to increase the number of patients participating in the study, increase doses and time of incubation, monitoring daily the effect to the drugs and study the influence of previous treatment as well as perform these studies in CLL cell lines. The methylation study needs to be made also in samples incubated with all four drugs in monotherapy (IC_{50}) and combination to compare with the results obtained in this study and analyse the influence of these epigenetic modulators in CLL cells. Finally, it is necessary to understand why ALL and CLL cells demonstrated to be more sensitive to the induction of apoptosis by histone deacetylase inhibitors, because this fact may be vital to develop better and more effective therapeutics for these two diseases.

This project, in a global way, represent a starting point for new studies involving epigenetic modulators in ALL and CLL diseases. Data showed promising results, and it may contribute to the beginning of a new era in ALL and CLL treatment, with the administration of epigenetic modulators as first-line treatment.

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